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CYTOLOGICAL STUDIES OF THE REACTION OF MYELINATED NERVE FIBERS TO SECTION OF THE NERVE[1]

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TWO PLATES (FIVE FIGURES)

Recorded observations of the properties and reactions of living peripheral nerves are meager. Hoerr ('36) critically reviews the work done on fresh nerve cells in relation to the existence of nurofibrillae. His criticism that "such cells must always be considered as dying cells rather than as living nerve cells" is applicable to similar studies of peripheral nerves removed from the body and studied fresh (de Renyi, '29).

Speidel ('32, '33) by means of a special ingenious technique made it possible for the first time to watch directly in the living organism the fundamental activities of nerve fibers over a considerable period of time. He extended these studies on tadpoles and salamanders to include phenomena of nerve irritation and recovery, degeneration and repair ('35). This investigation remains a classic in its field.

Because data on living mammalian nerves are meager and correlations between observations of living nerves and fixed nerves are fragmentary it was suggested that cytological studies combining quartz rod illumination of living nerves and cytological preparations of fixed nerves to elucidate the initial processes involved in the "axonal reaction" might constitute a significant contribution to the war research on nerve regeneration.

The procedures in this investigation included the examination of living nerves in frogs and rats, intercostal nerves of rats fixed in situ, cytological preparations of intact and cut sciatic nerves of rats and of cut sciatic nerves treated with vitamin gelatin. The experiments and

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[1] With the aid of the Mary E. Woolley Fellowship from Mount Holyoke College, South Hadley, Massachusetts, for which I wish to express cordial thanks.

I am greatly indebted to Prof. G. W. Bartelmez, who suggested the problem, for his kind aid and interest in this investigation.

I wish to express my gratitude to the Medical Departments of Eli Lilly and Co., Indianapolis, and Merck and Co., Rahway, New Jersey, for their generous cooperation in supplying vitamin products and valuable suggestions.

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observations to be described indicate that the first changes which appear when a myelinated nerve fiber is cut are largely due to imbibition of fluid; that these changes may be prevented or reduced by the application of a relatively inert, non-inflammatory, hydrophilic agent, such as dry gelatin.

LIVING NERVES OF FROG

Intact and cut nerve fibers were studied in situ in living frogs for periods of time varying from 15 minutes to 4 hours by means of the quartz rod illuminator (Knisely, '36) and a Zeiss binocular compound microscope with water immersion lens. The procedure was as follows:

After pithing the frog, an area of skin large enough to expose several abdominal nerves on the left side was carefully reflected without loss of blood. A small transverse incision was made through the body wall in the right lower quadrant parallel to the course of the abdominal spinal nerves and in such a way that there was a minimum of bleeding. (Female frogs with eggs were found to be preferable because of the thinner abdominal wall.)

A 1:100,000 solution of methylene blue in 0.7% salt solution at room temperature was allowed to flow slowly through the quartz cannula which provided the illuminating tip. (This proved to be a good light- and heat-filter as well as a vital stain.) The quartz tip was inserted into the small transverse abdominal incision and centered under a large left abdominal nerve with accompanying blood vessels. Care was taken not to injure the nerve in any way. A 0.7% salt solution was allowed to drip through a small cannula over the exposed abdominal musculature to prevent surface drying and to provide the fluid for the water immersion objective.

When the nerve was visualized under low power it was put under moderate tension by lowering the microscope stage or raising the quartz tip so that the blood flow was slowed but not stopped. The nerve fibers were then studied with the water immersion objective.

The nerve bundles of the intact abdominal wall appeared wavy ("sinuous"). The myelin sheaths were clearly refractile. Nodes of Ranvier could be seen. The axis cylinder usually appeared homogeneous but sometimes appeared "brushed" i.e., as though the axioplasm had been stroked by a fine brush (but that may have been due to diffraction of light by underlying myelin sheaths). There were no free spaces inside the neurilemma sheath. The myelin sheath was continuous and smooth. Neurilemma cells were not visualized. The field gradually stained with the methylene blue. The muscles, and connective tissue cell nuclei became progressively more deeply stained. The pigment cells became a nuisance. But neither the axis cylinders nor myelin sheaths became detectably stained. These observations were made on ten frogs and on several nerves in each frog.

When the nerve was cut the distal end, and the proximal end up to the first node of Ranvier (when it was identified), showed characteristic changes in the myelin sheath such as swelling, development of ''myelin figures'', disintegration. The axis cylinder appeared contracted and stained moderately with the methylene blue. These changes were rapid.

In the more central portion of the cut nerve the myelin sheaths gradually lost their refractile appearance as they became swollen and then invisible. A clear space developed between the axis cylinder and the region of the myelin sheath and the former stained progressively darker with methylene blue as it diminished in diameter. Finally the edges of the myelin sheath became rather intensely stained. These changes took place in 30 minutes to 3 hours. These observations were made on six frogs and on several nerves in each frog.

Because the changes in the central part of the cut nerve appeared to be hydropic (i.e. due to hydration), it was thought that the application of an inert hydrophilic agent to the cut end of the nerve might retard or prevent these changes. Dry white-label sheet gelatin was chosen for the purpose.

A cut nerve was visualized under the water immersion lens, a few millimeters proximal from the cut end. When the hydropic changes became apparent a wedge shaped strip of gelatin was inserted into the incision and in contact with the cut end of the nerve.

Gradually a refractile line appeared in the region of the invisible myelin sheath. The axis cylinder slowly lost its stain and the clear space between the axis cylinder and the myelin sheath disappeared. No times were recorded for these changes. These observations were made on two frogs and were repeated four times on one nerve by withdrawing and replacing the gelatin.

LIVING NERVES OF RAT

For the study of living mammalian nerve fibers the peroneal nerve of the young rat was chosen since the abdominal nerves undergo too great an excursion with respiration for continuous observation. In the young rat the peroneal nerve may lie superficial to the tibiofemoral joint and not so close to the large blood vessels as to prevent its being carefully freed.

After the animal had been injected intraperitoneally with a small amount of veterinary nembutal in solution, a small flap of skin was removed over the site of the peroneal nerve without causing any appreciable loss of blood. The nerve with its fascia was then carefully freed from the underlying structures for a distance of about 5 mm. The perineurium and accompanying blood vessels were not disturbed.

A perfusion bottle was filled with buffered mammalian physiological saline solution containing a small amount of calcium. This solution was warmed as it flowed through a U-tube immersed in a large volume of distilled water heated by a thermostatically controlled photographic immersion heater. The distilled water was heated to such a temperature that the saline which flowed out through the surface drip cannula varied in temperature from 33° to 40°C., depending on the rate of the flow.

For these studies a simplified home-made illuminator was used which produced so little heat that a quartz rod tip could be used in place of the quartz cannula tip and only the surface drip was required to prevent surface drying and to provide the fluid for the water immersion objective. This greatly facilitated the control of the temperature.

The rat was mounted on a draining tray on the adjustable stage of the microscope and the studies were made as for the frog nerve except that in some instances Janus green was used as a vital stain in place of methylene blue. The concentration of the dye was not determined. A few drops of a 0.1% solution of Janus green B in distilled water were added to the saline in the perfusion bottle until the density of color which produced the optimum vital staining was obtained.

Studies of the living nerve in the rat are much more difficult than in the frog. Temperature control is more important and more difficult. Optimum anaesthesia in rats is much more uncertain and variable in duration than is pithing in frogs. The individual variations in the peroneal nerve and its relations to the blood vessels constitute an operative hazard. Out of many attempts only six successful observations were made on rats but these were sufficient to confirm for the mammal some of the significant observations made on the frog.

After the initial shock the exposed nerve had a good circulation. The nerve fibers had smooth fine glistening myelin sheaths. The axis cylinders appeared transparent. With optimum concentrations of Janus green, the neurilemma cells became stained as did the perivascular muscle cells. Mitochondria were stained in both but none were visualized in the axis cylinder. The stained neurilemma cells made a continuous sheath around the myelin sheath.

When the nerve was manipulated by pinching or cutting, the local circulation of blood in the nerve was increased. After cutting the nerve the myelin sheath became invisible, the axis cylinder swelled and had a "brushed" appearance. Eventually the axis cylinders decreased in diameter and became stained with Janus green.

When the animal died and blood circulation stopped without cutting the nerve the myelin sheath swelled and the axis cylinder was constricted. The swelling of the myelin sheaths was maximal at the nodes of Ranvier with some formation of "myelin figures". The shrunken axis cylinders became stained with Janus green and the neurilemma cells were stained a diffuse blue.

NERVES OF RAT FIXED IN SITU

Young adult rats were anaesthetized with 0.1 cc. nembutal intraperitoneally. Incisions were made through the left abdominal wall, cutting abdominal spinal nerves. After 45 to 60 minutes the cut nerves were fixed in situ in the living animal by injecting, locally, through a hypodermic needle, about 5 cc. of the following mixture:

anhydrous acetone	4 parts
2% aqueous osmic acid	1 part
or	
neutral formaldehyde	9 parts
physiological salt solution	1 part

A small piece of abdominal wall containing the nerves was cut out and mounted in the fixative (lightly covered with a coverslip). The intact nerves of the opposite side were fixed in situ and treated in the same way. In some instances the preparations were treated as whole mounts, i.e., dehydrated in absolute alcohol, cleared in iso-safrol and mounted in balsam with a coverslip. In other instances the tissue was imbedded, sectioned at 3 to 5 μ and stained with phosphotungstic acid hematoxylin or aniline safranin.

In the whole mount preparations (figs. 1 and 2) the changes in the myelin sheath reflected those observed in the living cut nerve fibers. The intact nerves (fig. 1) presented a smooth fine myelin sheath uniformly blackened with osmic acid. No details were visible in the axis cylinder. The myelin sheaths of the cut nerves (fig. 2) were swollen and vacuolated and the space containing the axis cylinders appeared diminished in diameter.

In thin sections of such material it was evident that even in the intact nerves the myelin sheath was not homogeneous but presented a latticework appearance with areas more and less blackened with osmic acid. The diameter of the myelin sheath, however, was uniform for a given fiber and most of the axis cylinders filled the space within the myelin sheath and the neurofibrils were clearly visible. In serial sections of some intact nerves, in cross section, variation in the size of the axis cylinders within the sheath was noted. In some fibers the axis cylinder had shrunk away from the myelin sheath, leaving a clear space. In these instances the axis cylinders stained more intensely. This might have been due to incomplete fixation. This is characteristic of most histological preparations of central and peripheral nervous tissue.

In the sections of nerve cut 45 to 60 minutes before fixation the myelin sheath was highly vacuolated and varied in thickness along the course of one fiber, from almost completely filling the nerve tube to a thin line next to the neurilemma. The axis cylinder was condensed to a fine intensely staining line, and in most cases there was a large space between the axis cylinder and the myelin sheath.

INTACT SCIATIC NERVES OF RAT

Since the fixatives mentioned above were not suitable for studying the cytological details in large intact and cut nerves in imbedded and sectioned material, various other fixatives were tried. Of these a modified Maximow's fluid [2] was found to be most acceptable since it preserved the greatest number of cytological constituents in the relation in which they had been visualized by the quartz rod illuminator.

To secure specimens of intact nerves, the rat was lightly anaesthetized with nembutal following which the sciatic nerve was exposed and freed, avoiding loss of blood. Then a 2-mm. segment of the nerve was cut out and immersed immediately in the fixative.

In this and the following series the nerves were iodized during the process of dehydration, imbedded in paraffin, serially sectioned at 2 to 6 μ and every fifth slide of each series stained by Hoerr's modification of the Altmann-Bensley Aniline acid fuchsin-methyl green method for mitochondria, i.e. 20% analine acid fuchsin — 5% phosphotungstic acid — 1% methyl green.

With this fixation and staining the axis cylinders stain palely with the green dye, the neurofibrils a little more intensely than the axioplasm. Along the neurofibrils there are filaments and granules which stain rather intensely with the red dye. These filaments are thicker than the neurofibrils and resemble mitochondria but may be thickenings along the neurofibrils (fig. 3). The identity of these fibrils as mitochondria has not been established since, as yet, mitochondria have not been visualized in the living peripheral nerves stained with Janus green and studied with the quartz rod illuminator.[3]

---

[2] Zenker-formol-osmic (Bensley and Bensley, '38). Bensley's aqueous chrome-sublimate, 9 parts (modified Zenker's fluid); redistilled neutral formaldehyde, 1 part; 2% aqueous osmic acid, 1 part.

The unblackened portion of the myelin sheath stains golden to red. The reticulum stains a bright blue green. The neurilemma cells are well fixed and stained, their nuclei and especially the nucleoli and mitochondria brilliantly stained with the red dye, the cytoplasm with a combination of the two dyes. The endoneurium cells are not as well fixed, their nuclei more condensed stain deeply with a mixture of both dyes. By these characteristics they may be distinguished from neurilemma cells. Endothelial and muscle cells of the blood vessels are well fixed and their mitochondria brilliantly stained. The myofibrils of the smooth muscle cells tend to stain with the green dye. On the whole there is remarkably little shrinkage of the nerve fibers and neurilemma cells with this method, and in thin sections the cytological details are well visualized.

In nerves cut or cut and treated with vitamin gelatin the delicate differentiation afforded by this method is invaluable. Dispersed myelin is readily distinguished from coagulated axioplasm and the latter from end bulbs. Fine nerve fibers are easily differentiated by their brilliantly stained granules and filaments (mitochondria?). Neurilemma cells can be differentiated and identified by their nuclear and cytoplasmic characteristics. Mitotic figures are clearly defined even to the spindles and centrosomes. Reticulum is clearly differentiated. Newly forming myelin is brilliantly stained with the fuchsin.

CUT SCIATIC NERVES OF RAT

Sciatic nerves were exposed aseptically under nembutal anaesthesia and a 2-mm. segment of each nerve was removed. The wounds were closed with skin clips and the animal allowed to recover. Twenty-four hours after operation pieces of the nerve just central and distal to the cut were removed and immediately immersed in the fixing fluid.

In these preparations (fig. 4) the cut nerve fibers showed a protein coagulum extending beyond the cut end of the fiber. The myelin sheaths at the cut end were in various stages of disintegration. Some axis cylinders were shrunken and condensed, others appeared to be swollen masses of protein coagulum and still others were dispersed granular masses. Rarely a fiber containing a swollen axis cylinder with fuchsin staining granules was found. One instance of new branches underneath the neurilemma in the nerve tube at the cut end was found.

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[3] Mitochondria have since been vitally stained with Janus green in the non-myelinated axis cylinders of the terminal branches of the mylohyoid nerve and their motor end plates, in the frog, and observed with quartz rod illumination.

More centrally there was considerable variation in the appearance of the fibers. Some axis cylinders appeared coagulated, some shrunken, some almost normal. The myelin sheaths also showed varying degrees of vacuolization. In this region many of the neurilemma cells were swollen and in these the nucleoli were large and prominent.

CUT SCIATIC NERVES TREATED WITH VITAMIN GELATIN

For the study of the effect of gelatin on the cut sciatic nerve of the rat a mixture of gelatin and vitamins was used in most of the experiments so far examined. The incorporation of vitamins into the gelatin was incidental to a study of wound healing which was being conducted at the same time. The specific effects of vitamins locally applied to the cut nerve and their significance in nerve regeneration are the subject of further analysis and study and will not be discussed in this preliminary report. The method used was as follows.

A 20% gelatin (containing most of the B complex and ascorbic acid) with a pH of about 5, was made into fibrils, micro-tubules, or large tubes in such a way that the molecules were presumably linearly arranged in the long axis of the fibril or tube. These were dried and then sterilized in 95% alcohol for several days.

Sciatic nerves were operated on as for the untreated cut nerves and the vitamin gelatin placed between the cut ends. After 24 hours the portion of the nerve so treated was removed under nembutal anaesthesia and immediately immersed in the fixative.

In these preparations (fig. 5) there was very little coagulated protein at the cut end of the nerve but many end bulbs and growth cones occupied this zone. Some of the nerve tubes close to the cut end contained axones which were already branching within the myelin sheath. Many nerve tubes contained end bulbs. There was remarkably little swelling and breaking up of the myelin even at the cut end.

More centrally the tissue spaces were enlarged. The axis cylinders were large and the fuchsin-staining filaments very conspicuous along and between the neurofibrils. The myelin sheaths appeared normal. There was no marked swelling of the neurilemma cells in this region.

DISCUSSION

Studies of living nerves by quartz rod illumination confirm for the adult frog and the rat some of the observations on amphibian larvae reported by Speidel ('33 a, '35 a), namely, the swelling of the myelin sheath, vacuole formation between myelin sheath and axis cylinder, shrinkage of the axis cylinder, and the appearance of a neurofibrillar structure within the axis cylinder, after sectioning or injury of the nerve fibers.

Whether vital staining of the axis cylinder of myelinated nerve fibers with methylene blue is dependent on changes due to injury of the nerve fiber is a question raised but not solved by these experiments. The early changes which occur in the nerve fiber whether the animal simply dies or the nerve is cut may account for the pictures that are commonly seen when nerves are removed from dead bodies or when fixation has been delayed.

The swelling and change in the refractility of the myelin sheath with the subsequent shrinkage or condensation of the axis cylinder early after section of the nerve suggests imbibition of fluid by the myelin and possibly a physical and chemical reorganization within it. Whether this fluid is derived from the axis cylinder as Speidel suggests, or from the tissue fluid because of disturbances in the delicate equilibrium in water balance is not clear. Undoubtedly minute variations in these reactions depend on a number of factors such as blood supply, size of the fiber, thickness of the myelin sheath, the physical and chemical properties of the myelin sheath, etc.

Sometime within the first 24 hours after cutting the nerve fibers the early changes apparently become irreversible at the cut end, at least as far as the first proximal node, and both the myelin and the axone disintegrate. The water balance is apparently restored central to the cut end before extensive disintegration of the myelin occurs in most fibers. The gelatin of the injured axone (de Renyi, '29) the formation of a clot at the cut end, and the influence of the perikaryon extending down the axone may be factors in restoring the water balance.

The experiments with gelatin seem to indicate that when dry gelatin is applied to the cut end of a myelinated nerve fiber the early changes which ordinarily appear after section of the nerve may be reversed or prevented; and that if these early changes are inhibited, the later disintegration of the myelin — and axone is reduced. How gelatin prevents or retards these changes can only be surmised. Apparently the gelatin restricts the imbibition of water by the myelin thus preventing it from swelling and disintegrating at the cut end and so leaving the nerve tube open. This mechanical effect could explain the early swelling, extension, growth and branching of the axone between the cut end and the first proximal node within the first 24 hours after section of the nerve. Whether these effects of gelatin can influence the regeneration and reconstitution of a severed nerve is a problem for further study.

SUMMARY AND CONCLUSIONS

The immediate effects of sectioning a myelinated nerve fiber can be observed by means of quartz rod illumination in the living animal. These changes can be checked and recorded in permanent preparations by fixing the nerve in situ in the living animal. The later effects of severing the nerve can be determined by cytological preparations of intact and severed nerves.

The sequence of events is as follows:

1. The cut ends of the nerve fibers swell, the myelin takes up water, exhibits ''myelin figures''; the axone may or may not retract.
2. More centrally the nerve tubes swell, the myelin loses its high refractility, and a clear space develops around the axis cylinder. The axone subsequently shrinks.
3. A protein coagulum extends from many of the cut nerve fibers beyond the zone of section. In many instances fragments of axones, neurilemma cells and myelin may be found in this coagulum. In many fibers the nerve tube is completely occluded by dispersed myelin and axioplasm, at least so far as the first proximal node of Ranvier.

When dry gelatin is applied to the cut ends of the nerve fibers these changes are prevented or reduced and growth and branching of the axones at the cut end is observed within 24 hours.

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PLATE 1

EXPLANATION OF FIGURES[4]

These figures are camera lucida drawings made at table level with a 2-mm. Zeiss apochromatic oil immersion objective lens with 10 x oculars, and represent a magnification of about 1080 × for each figure after ½ reduction.

1 Two fibers of an abdominal nerve of the rat fixed in situ by stick injection with acetone-osmic, removed into the fixative, subsequently dehydrated with alcohol, cleared in iso-safrol and mounted in balsam as a whole mount. Note the smooth outlines of the myelin sheaths, their uniform distribution and density.

n, node of Ranvier. m, myelin sheath. coll, collateral branches.

2 One fiber of an abdominal nerve of the rat cut for 45 minutes and then treated like the preceding. Note the swelling of the whole fiber, the irregular outline of the myelin sheath, its vacuolation and uneven distribution, especially at the node of Ranvier.

n, node of Ranvier. m, myelin sheath.

3 One fiber of a rat sciatic nerve fixed in redistilled neutral formol – Zenker – osmic fluid, imbedded in paraffin, sectioned at 4 μ and stained with aniline acid fuchsin – phosphotungstic acid – methyl green. The fiber lies deep in the sciatic nerve and shows the effects of delayed or incomplete fixation, especially at the node of Ranvier, namely: vacuolization and swelling of the myelin sheath, slight swelling of the neurilemma cells and the constriction of the axone at the node. These effects resemble the changes which appear in the living nerve when it is cut but to a lesser degree. Note the fine neurofibrils and the mitochondria-like filaments.

n, node of Ranvier. e, endoneurium. s, cytoplasm of neurilemma cells. a, axioplasm with neurofibrils. m i, mitochondria-like filaments and granules. m s, myelin sheath.

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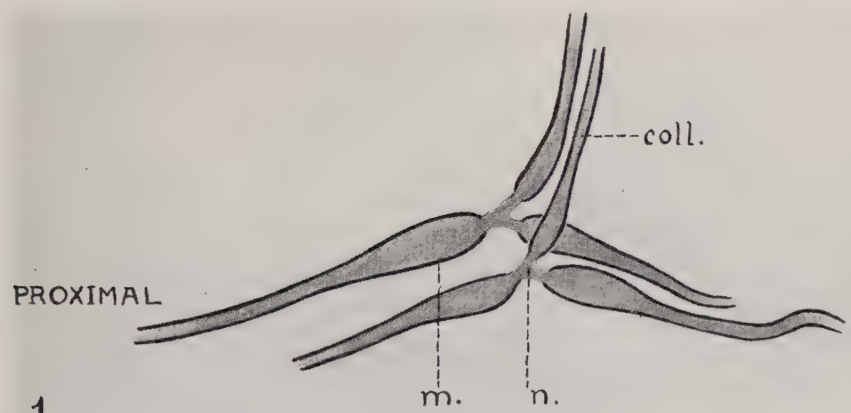
[4] I am indebted to Miss Agnes Nixon for the drawings.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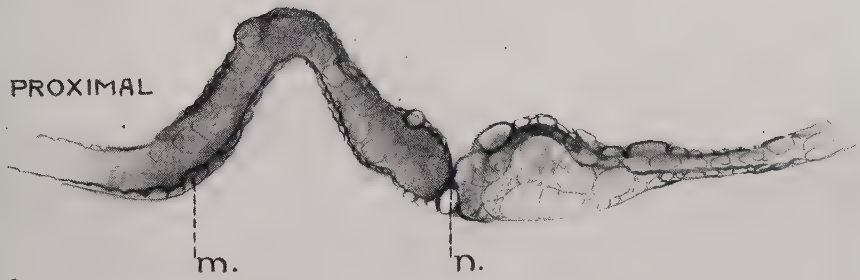
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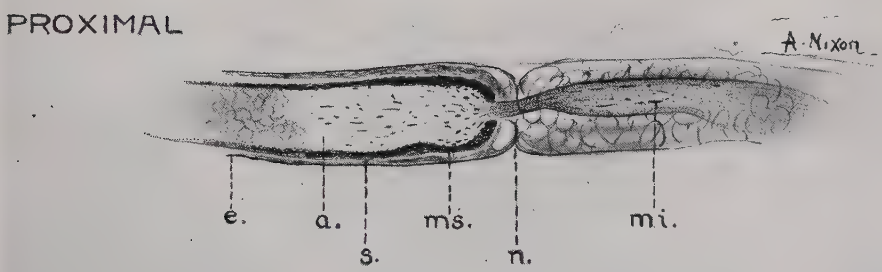
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PLATE 2

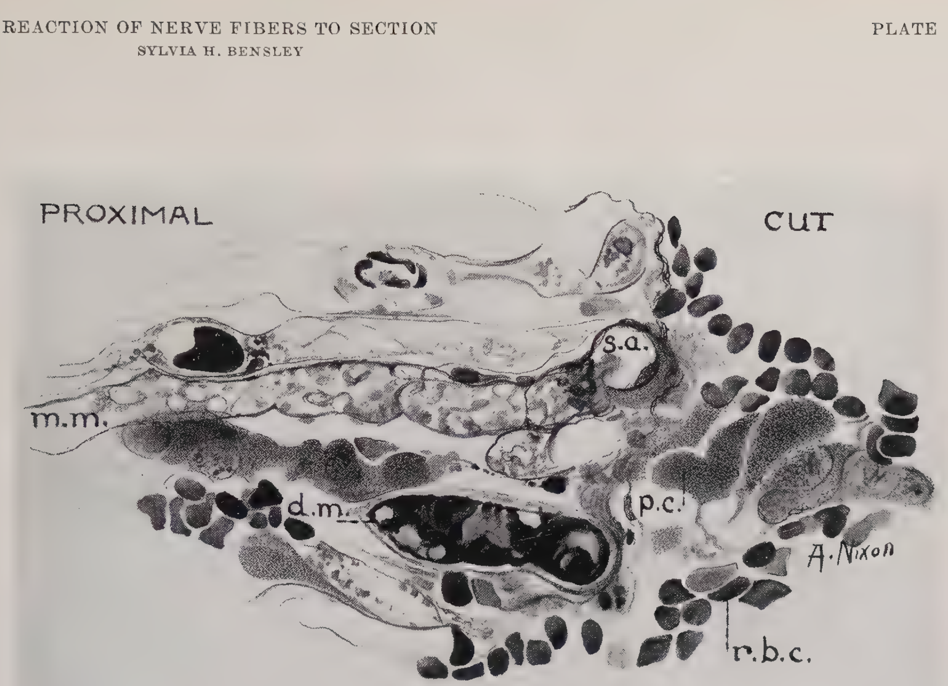
EXPLANATION OF FIGURES

4 Cut end of a rat sciatic nerve removed 24 hours after section, fixed in redistilled neutral formol – Zenker – osmic fluid, imbedded in paraffin, cut at 4 μ, stained with aniline acid fuchsin – phosphotungstic acid – methyl green. Compare size of fibers with figure 3. Note the protein coagulum at the cut end, the shrunken axone, the occlusion of the nerve tube with degenerating myelin.

p c, protein coagulum extending beyond cut surface. r b c, extravasated red blood cell. m m, myelin sheath exhibiting myelin figures. d m, dgenerating myelin. s a, shrunken axone.

5 Cut end of a rat sciatic nerve, treated with vitamin gelatin, removed 24 hours after operation and treated like the preceding. Compare size of fibers with figures 3 and 4. The increase in size here seems to be due to the presence of large end bulbs, and branches of axones in the cut ends of the nerve tubes. Note the absence of protein coagulum at the cut end, the open nerve tubes and the relatively small amount of degenerating myelin.

m m, myelin sheath exhibiting myelin figures. d m, degenerating myelin. e b, end bulb of axone within an otherwise empty nerve tube. l, leucocyte within nerve sheath. r b c, extravasated red blood cell. s b, sub-neurilemmal branches of axone. m a c, connective tissue macrophage.


PROXIMAL
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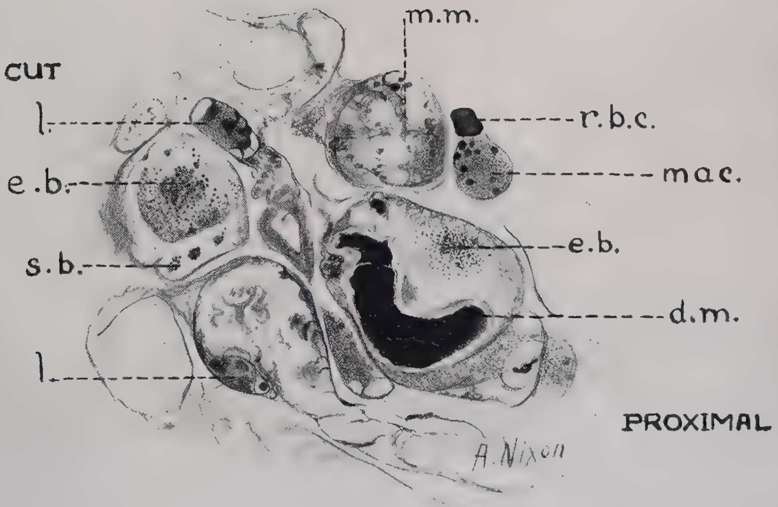
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5

THE EXTERNAL CAROTID ARTERY OF THE RHESUS MONKEY (MACACA MULATTA)

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ONE FIGURE

In examining the literature I found that no detailed information on the external carotid of the rhesus monkey (Macaca mulatta) was available. The wide use of this animal for experimental purposes made it seem worthwhile to place these data on record.

For this study four specimens were dissected. The vessels were washed out with saline and perfused with a formol-acetic embalming fluid. The arteries were injected through the left ventricle of the heart with a chrome yellow starch mass prepared as described by Hyman ('22, p. 371). The finer arteries were dissected out under a binocular dissecting microscope.

I am indebted to Dr. James M. Sprague and Dr. W. L. Straus, Jr., Department of Anatomy, Johns Hopkins University, for the criticism and advice they have given in the preparation of this paper, and to Drs. David Bodian and Howard Howe, Poliomyelitis Research Center of the Johns Hopkins University, who supplied the animals for study.

The vascular supply of the primate head has been studied in many species during the last century. Theile (1852) studied four specimens of Simia inuus (Macaca sylvanus) and gave a passing comment on the presence of a foramen for the recurrent meningeal branch of the lachrymal artery in skulls of Simia maimon (Papio sphinx) and Macacus (Macaca sp.). Rojecki (1889) reported on a series of dissections of Macacus cynomolgus (Macaca irus) and M. sinicus (M. radiata). In 1899 Tandler gave excellent accounts of Cynocephalus (Papio) and Semnopithecus as well as Hapale and Ateles, but he did not dissect a macaque. His later paper ('02) described a number of other mammals but made no further contribution to a knowledge of primates. Hafferl ('33) summed up the literature to that date following Tandler's outline in briefer form, but added nothing relating to the primates. In the same year, Lineback, in Hartman and Straus' "Anatomy of the rhesus monkey'', published an account of the vascular system which gave only cursory and incomplete consideration to the blood supply of the head. Ask-Upmark ('35), in his study of the carotid sinus and the cerebral circulation, commented on the fact he found Macaca mulatta to have an arterial arrangement and cerebral blood supply essentially the same as in man, differing only in that the bifurcation of the common carotid is situated nearer the base of the skull.

The extracranial carotid system of the rhesus monkey, as shown in the accompanying figure, basically resembles the external carotid in man, but differs in several respects:

1. The carotid bifurcation, as mentioned by Ask-Upmark, is higher in the macaque, being at the level of the hyoid bone. The external carotid is slightly larger than the internal carotid. This difference did not exceed 0.8 mm. in two specimens measured a few millimeters above the bifurcation.

2. The superior thyroid, external maxillary and lingual arteries are given off at approximately the same level in the macaque, either as three separate trunks (one specimen), or as a superior thyroid and a glosso-maxillary artery (two specimens), or as a single, common vessel (one specimen).

3. A common posterior auricular occipital trunk supplies both auricular and occipital fields, and several sterno-cleido-mastoid rami come directly off the external carotid trunk. In two specimens a small branch of the occipital artery was looked for and found entering the skull along an emissary vein through the foramen lacerum spurium. A small posterior auricular vessel is given off by the external carotid which supplies a constant stylomastoid artery and a variable proportion of the posterior auricular field. In three of the four specimens examined this contribution was small.

4. The internal maxillary artery is larger and more sinuous. Lineback ('33) reported the middle meningeal to be its chief branch; in my series, however, the posterior deep temporal was unquestionably its largest single branch. In all four of my specimens the external carotid continued as the internal maxillary rather than as the superficial temporal as he indicated.

5. The superficial temporal is relatively smaller.

6. The middle meningeal is relatively smaller and is given off from the internal maxillary artery at more of a right angle.

7. The posterior deep temporal artery is larger, in one case really a bifurcation of the internal maxillary. The anterior deep temporal

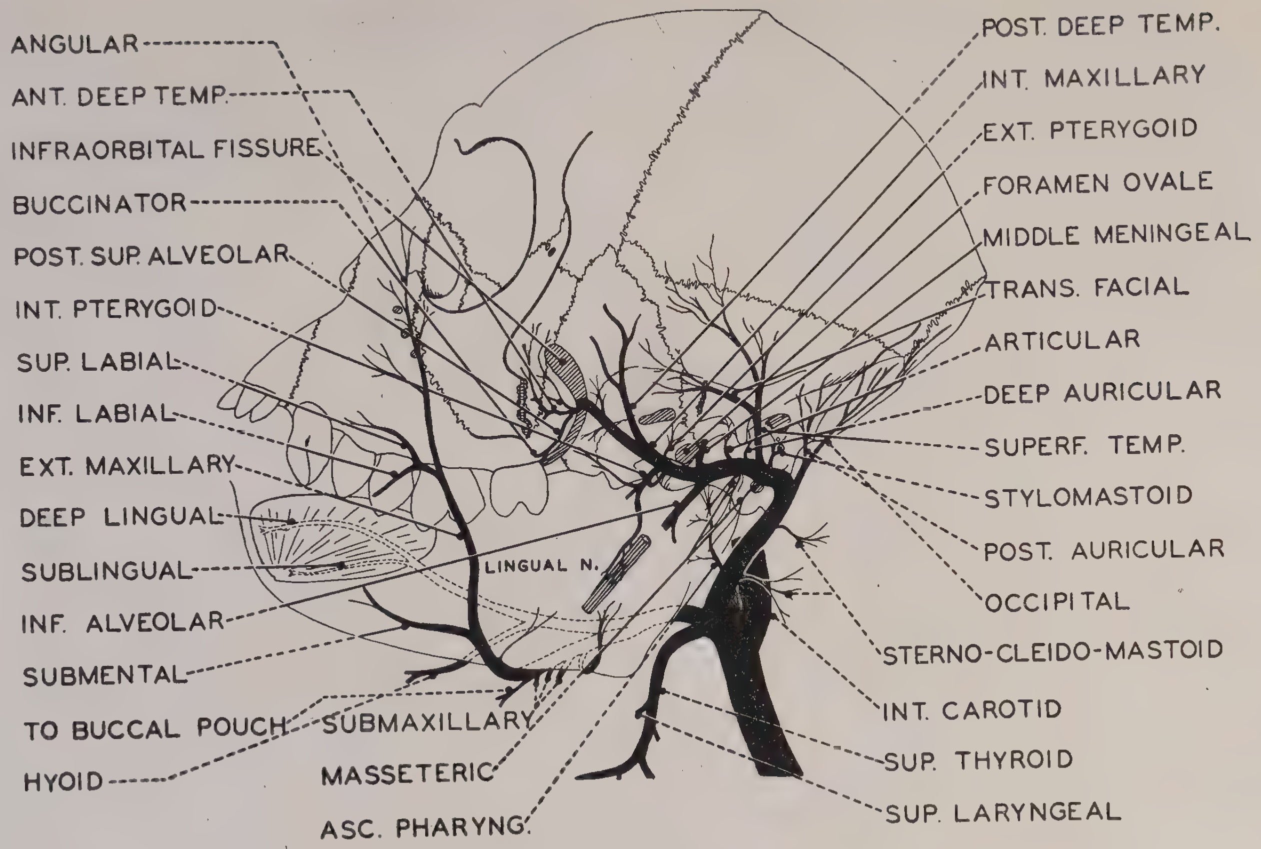
ANGULAR
ANT. DEEP TEMP.
INFRAORBITAL FISSURE
BUCCINATOR
POST. SUP. ALVEOLAR
INT. PTERYGOID
SUP. LABIAL
INF. LABIAL
EXT. MAXILLARY
DEEP LINGUAL
SUBLINGUAL
INF. ALVEOLAR
SUBMENTAL
TO BUCCAL POUCH
HYOID
SUBMAXILLARY
MASSETERIC
ASC. PHARYNG.
LINGUAL N.
POST. DEEP TEMP.
INT. MAXILLARY
EXT. PTERYGOID
FORAMEN OVALE
MIDDLE MENINGEAL
TRANS. FACIAL
ARTICULAR
DEEP AURICULAR
SUPERF. TEMP.
STYLOMASTOID
POST. AURICULAR
OCCIPITAL
STERNO-CLEIDO-MASTOID
INT. CAROTID
SUP. THYROID
SUP. LARYNGEAL

Fig. 1 Lateral view of the left external carotid artery and its branches in the rhesus monkey. Foramina are cross hatched. Approximately natural size.

field is much smaller and supplied by several small vessels with a short common trunk.

These data are similar to those for other macaques, but there are certain differences. Rojecki (1889) found M. irus and M. radiata to have only a slight angulation at the level of the occipito-auricular branch, with the internal maxillary turning more sharply to the horizontal at the level of the superficial temporal. Theile's (1852) findings on M. sylvanus correspond closely with mine on M. mulatta (this is based on his textual material alone since his only illustrations were of the leg and hand). The one difference is a laryngeal vessel which he found was given off directly from the external carotid. In the rhesus monkey the larynx is supplied by the superior thyroid artery.

These Old World forms differ markedly from the New World marmosets, Hapale penicillata and H. jacchus Kuhl, and the spider monkey, Ateles paniscus. In the marmoset (Hapale jacchus Kuhl), Beattie ('27) found the internal maxillary artery to be small, the superficial temporal large. Tandler (1899) found that Hapale penicillata had no middle meningeal given off from the internal maxillary, but that its field was supplied by a recurrent branch of the lachrymal artery. In Ateles paniscus, Tandler found a small transverse facial artery given off directly from the external carotid, and also a large occipital vessel. He found a large transverse facial artery in Papio sphinx and Semnopithecus sp. which is given off directly from the external carotid, whereas in the rhesus monkey it branches from the superficial temporal. With regard to other branches of the external carotid Hapale, Ateles and Macaca agree with one another and with man.

In mammals, the fundamental vascular pattern of the head is formed by the external carotid, internal carotid, and stapedial arteries. Typically, the internal carotid (derived from the dorsal aorta) is closely associated with the developing brain. The external carotid (derived from the ventral aorta) is associated with the hyoid and lingual regions, including the thyroid. The stapedial artery (derived from the internal carotid at or near the level of the second or hyoid aortic arch) supplies the dura, the orbital and the maxillo-mandibular areas. In all mammals the internal carotid passes forward between the base of the skull and the pharynx. In the mammalian embryo the stapedial artery passes outward over the tympanic cavity, pierces the stapes, and then runs forward through the cranio-quadrate passage to the orbit.

Goodrich ('30) believed that phylogenetically the external carotid gradually takes over the distribution of the stapedial. It was his interpretation that the mandibular artery is primitively stapedial in origin.

The mandibular artery is taken over by the external carotid in crocodiles, leaving a stapedial artery with its supra- and infraorbital rami in the adult. Uniquely enough this same pattern is exhibited by the adult rat. In man and most other placental mammals all three branches are taken over by the external carotid.

Tandler (1899), on the other hand, held that the mandibular ramus is a primary branch of the external carotid which is taken over by the stapedial artery in the insectivores in a manner similar to that in which the carotids take over the terminal rami of the stapedial in man. In the latter, the external carotid takes over the distribution of the inferior ramus, and the internal carotid, the distribution of the superior ramus. Tandler based his conclusion on the fact that the mandibular ramus in mammals, represented by the inferior alveolar artery, is given off from the primitive external carotid trunk, and lies lateral to the third division of the trigeminal nerve, whereas the inferior ramus of the stapedial artery runs medial to this nerve. A vestige of the superior ramus proper is found in most simian species including man, as a recurrent twig from the lachrymal artery which in turn penetrates the posterior superior lateral angle of the orbit and anastomoses with the anterior portion of the middle meningeal artery. The proximal portion of the middle meningeal is thus formed by the orbital branch of the inferior ramus of the stapedial artery.

Davis and Story's ('43) excellent presentation of the carotid circulation in the domestic cat demonstrated the development of external carotid dominance in carnivores, terminating in a complete occlusion of the proximal internal carotid in the cats. The internal carotid field is supplied by three anastomotic vessels listed in order of caliber: an internal maxillary branch to the circle of Willis, a middle meningeal branch to the distal internal carotid, and an ascending pharyngeal continuing into the distal internal carotid. Thus the two carotid systems are potentially collateral to one another. The presence of important proximal branches in the external carotid and the lack of any such vessels in the internal carotid system minimizes reciprocal variation toward internal carotid dominance.

In the rhesus monkey, as in man, the external carotid supplies the skin, muscles of mastication and facial expression, and the meninges of the brain. The power of the muscles of mastication is reflected in their ample blood supply. The relatively smaller brain in the macaque accounts for its smaller internal carotid, as well as middle meningeal and superficial temporal, rather than because of any support of the internal carotid circulation by external carotid anastomoses.

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THE ORIGIN OF OVA AND FOLLICLE CELLS FROM THE GERMINAL EPITHELIUM OF THE OVARY OF THE ALBINO RAT AS DEMONSTRATED BY SELECTIVE INTRAVITAL STAINING WITH INDIA INK[1]

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TWO PLATES (TWELVE FIGURES)

Since the early work ('00) of von Winiwarter describing postfetal ovogenesis in rabbit and man, several methods have been employed to test the validity of the concept.

By selection of closely graded series of stages, numerous investigators have introduced evidence of the postpartum formation of ova. These reports have been critically reviewed by Kingsbury ('38). One of these, Arai ('20), after making extensive counts of the total number of ova present at different ages in rats, concluded that ovogenesis was a continuous process. Allen ('23), using mouse ovaries produced evidence of the cyclic nature of the process, initiated by mitoses in the germinal epithelium. Bagg and Papanicolaou ('24) were able to accelerate ovarian activity in guinea pigs by irradiation of the thyroid gland, and found ova, follicle cells, interstitial and lutein cells all were derived from the germinal epithelium. Allen and Creadick ('37) and Allen, Smith and Gardner ('37) by using colchicine in mice more clearly demonstrated mitosis in the germinal epithelium and found convincing evidence of cyclic proliferation of ova from the germinal epithelium, the peak appearing at estrus. Schmidt and Hoffman ('41) using this technique demonstrated such activity in guinea pig ovaries, only part of which was thought to be concerned in ovogenesis.

In another approach Long ('40) reported abundant growth of germinal epithelium in tissue cultures of bits of mouse ovaries. He further demonstrated the formation of ova and follicles from this cultured epithelium.

Marx ('41) using rats' ovaries stimulated by injection of gonadotropic extracts, found no evidence of proliferation of ova from the germinal epithelium except in the region of insertion of the suspensory ligament, leading him to conclude that the fimbria of the oviduct acted as an inductor for ovogenesis. Previously ('37) Pincus and Enzman found no evidence of ovogenesis in a large series of ova from sexually mature rabbits. Kingsbury ('38) found evidence of degeneration in the cortical zone of prepubertal cat ovaries, but not as extensive as earlier reported. Even when degeneration was extensive, large numbers of ova remained. He found no adequate evidence that ova were derived from the germinal epithelium either in the prepubertal or sexually mature cat. Cooperwaite ('25) found that young follicles were apparently formed in so-called ''germinal valleys'' in the ovaries of pre- and postpubertal rats because mitotic changes did not occur in the central cells of these follicles. Most investigators do not consider this objection valid.

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[1] A preliminary report of this investigation appeared as an abstract read by title at the meeting of the American Association of Anatomists, April 1, 2, 3, 1942 (Anat. Rec., vol. 82, no. 3, p. 81).

Considering the fact that quite early in life the ovary and part of the oviduct of the rat becomes partially or completely separated from the general peritoneal cavity by the periovarian membrane, a fold arising from the broad ligament, we conceived the possibility of injecting small quantities of an intravital dye into this bursa ovarica so that it might be selectively stored as granules by the germinal epithelium and only those mesothelial cells lining the rest of the bursa. Allowing a suitable time lapse any parenchymatous cells of the ovaries which contained dye granules could almost certainly be said to have been derived from the germinal epithelium, particularly in view of the very small amounts of dye introduced. and its localization in the bursa obviating generalized intravital staining.

MATERIALS AND METHODS

The albino rats used were taken from an inbred strain maintained by the department. The standard diet was reinforced with wheat germ and cod liver oil. Ages of selected virgin females varied from 1 to 12 months.

Higgins India ink was chosen for injection to avoid the possible factors of greater diffusibility and toxicity of colloidal dyes in the concentrations necessary to stain the germinal epithelium. The relatively large carbon particles were thought less likely to penetrate the epithelium to the underlying stroma and to be relatively inert and less disturbing to the physiological processes in those cells containing them.

Animals were anesthetized with sodium nembutal, reinforced when necessary by amounts of ether. Using sterile technique the abdomen was opened by midrectus incision so that either ovary and its surroundings could be reached and delivered to the surface. The ink was injected into the periovarian space through a steel needle, or, in very young animals, a glass micropipette. The needle or pipette was pushed through the fat body before penetrating the space, allowing for moderate distension without visible extravasation. In no case did the injected amount exceed 0.1 cc. without leakage. When leakage occurred the excess was absorbed by previously placed cotton sponges so that none escaped into the general peritoneal cavity. When a four to one dilution of India ink in saline was used no dye was found in the bursa and discoloration of the luminal surface of the oviduct indicated escape of the excess by this route.

In twenty-one individuals India ink was introduced into the periovarian space and from 1 to 130 days allowed to elapse before removal of the ovaries for study. In a few cases single ovaries were removed with a variable time interval intervening, but in most cases the animal was killed and the tissues fixed by intravascular injection followed by immersion. Methyl benzoate containing 4% nitrocellulose was used as the clearing agent and 56° paraffin for infiltration and embedding. Heidenhain's azan modification of Mallory's connective tissue stain was used routinely in order to avoid confusion between carbon inclusions and chromatin particles.

Twenty-four hours after injection of ink into the bursa, practically every cell of the germinal epithelium contained carbon inclusions, either in infranuclear clumps or in a perinuclear rosette (fig. 1). The cells of the lining epithelium of the Fallopian tubes were never found containing dye inclusions. The peritoneal mesothelial cells in the lining of the bursa exposed to the ink also contained inclusions in smaller amounts than those of the germinal epithelium. In addition to these locations sharply delimited masses of heavily laden macrophages were frequently found in the fat body adjacent to the ovary through which the injection needle or pipette had been introduced. Occasionally similar smaller masses of macrophages packed with carbon were found in the ovarian stroma, indicating that the injection needle had penetrated the germinal epithelium. As the technique improved such subepithelial masses were no longer found. In no case were inclusions found in definitive ova, follicle cells, or lutein cells 24 hours after local application.

As a control for the method two animals were heavily stained intravitally, one with India ink and the other with trypan blue, by intraperitoneal injections repeated twice daily over a period of 5 days. Subsequently neither granulosa cells, ova, nor lutein cells contained dye inclusions. Interstitial cells segregated small amounts as did other stromal elements. The germinal epithelium contained a considerable amount of dye and a few inclusions were noted in very small follicles lying immediately under the epithelium. As the injections continued over a period of 5 days and tissues were removed on the sixth day it seemed certain that these very small follicles had been newly formed during the period of injection.

As a further control measure normal ovaries comparable to those of the experimental series were prepared and studied for evidences of ovogenetic activity. In each case features observed in the experimental material were duplicated in the controls.

After selective segregation of carbon in the germinal epithelium ovaries were removed after time intervals varying from 1 to 130 days. If during these intervals ova and follicle cells were being formed from the germinal epithelium, one would expect to find these elements lying deep to the surface and containing carbon inclusions originally confined to the surface cells.

RESULTS OF EXPERIMENTS

The immediate response to injection of India ink into the ovarian bursa consisted principally of a mild inflammation. Twenty-four hours after injection a number of free cells were present in the periovarian space, a definitely increased number of leucocytes in the neighboring engorged vascular channels and a considerable number of segmented neutrophiles infiltrating the tissues of the ovary and periovarian membrane.

The free cells in the bursa were in most cases so heavily laden with ink as to obscure nuclear details. By comparison with those not so well filled it may be presumed that these consisted chiefly of typical macrophages with occasional segmented neutrophiles. Intermediate stages were present indicating the origin of many of these macrophages from the neighboring peritoneal mesothelium and from the germinal epithelium. Macrophages were recognized in the subepithelial connective tissue of the ovary, but rarely contained inclusions. The neutrophilic infiltration was largely confined to the tunica albuginea and immediately subjacent region. Both macrophages and neutrophiles lying on or between germinal epithelial cells had stored dye.

Careful examination of the germinal epithelium and subjacent tissue revealed a few examples of the initial phases of ovogenesis. Several large clear cells with large dark staining vesicular nuclei, the first indications of germinal potency, were found in the epithelium, each containing segregated dye (fig. 2). Ink granules were seen in another somewhat larger cell with clear cytoplasm and a large dark staining vesicular nucleus which lay slightly deep to the general level of the germinal epithelium (fig. 3). Another such potential ovum was observed still deeper in the epithelium covered by a somewhat flattened potential follicle cell which was in turn covered by typical epithelial cells (fig. 2). Both the prospective ovum and follicle cell contained cytoplasmic ink inclusions.

In all ovaries examined there were cleft-like extensions from the surface with the underlying tissue between rapidly growing parenchymal structures lined by germinal epithelium corresponding to the ''germinal valleys'' of Cooperwaite ('25). Along the sides, but most frequently at the base, of these epithelial pits or invaginations were occasionally found samples of the ''in situ'' differentiation of ova, most of which had included dye. A typical example of one of these developing ova is shown in figure 4.

No further evidences of new formation of ova in follicles following the introduction of dye were found at this period. Although one animal was killed at mid-estrus no mitotic figures were seen in the epithelial cells of any ovaries in this group. It is quite probable that the presence of the dye had some depressant effect on ovogenetic acivity.

In material sectioned between 1 and 7 days following injection of ink, there appeared a gradual subsidence of the inflammatory reaction as shown by a progressive decrease in the number of phagocytic cells in the periovarian space and the immediately surrounding tissues. As the exudate in the space receded clumps of macrophages in a fibrin network became invaded by fibroblasts from the periovarian membrane or the ovarian stroma and later were covered by typical peritoneal mesothelium or germinal epithelium, thus accounting for the occasional clusters of dye-laden macrophages just deep to the germinal epithelium or peritoneal mesothelium after an interval of 6 or 7 days. In the ovary these masses protruded from the general surface. Such clusters were also found in experiments extending over longer periods, but for the duration of these experiments apparently no dye was released from these sources.

As the interval between vital staining and sectioning increased there were increasing evidences of ovogenesis involving the dye-laden germinal epithelium, the various processes observed corresponding to those described earlier. We again failed to find mitoses in the germinal epithelium. The other parenchymal tissues of ovaries removed from experimental animals during this period were comparable to those of ovaries from controls of the same age.

After a period of from 1 to 8 weeks following intravital staining in addition to numerous further examples of direct differentiation of ova from the "impregnated" surface epithelium three other methods of ovogenesis were observed.

The first of these processes consisted in a thickening and wrinkling of the germinal epithelium to two or three layers of cells. The underlying connective tissue apparently pushed into thickened epithelium, in some places giving rise to villous projections in between which were rather deep crypts. Masses of undifferentiated dye-laden cells, identical with those on the surface, apparently split off from the depths of some of these crypts. As these masses became more widely separated from the surface one central cell enlarged into a primordial ovum, the remaining three or four cells flattening out to become follicle cells. The presence of ink granules in these differentiating cells leave no doubt of their origin from the germinal epithelium (fig. 5).

In some regions where the germinal epithelium was thickened and villous folds of the surface were seen, the intervening crypts had extended for some distance obliquely into the underlying stroma. At or near the end of these ingrowths were frequently found small groups of lightly stained cells containing dye inclusions similar to those just described (fig. 6). Moderate enlargement of some of the cells in these crypts indicate ovogenetic potentiality.

The third mechanism by which cells of the germinal epithelium may reach a subepithelial position and later differentiate into ova and follicle cells is through the downgrowth of solid cords of cells from the surface into the stroma. Columns of pale staining cells with numerous ink inclusions were found lying nearly parallel and deep to the tunica albuginea. In each case direct connection with the epithelium could be demonstrated (fig. 7). The course taken by the fibers in the tunica albuginea apparently influenced the direction of growth of the epithelial tubes and cords. The germinal epithelium was not noticeably increased in thickness in regions where the downgrowth of an epithelial cord had occurred, as seen in the other two instances.

After these cords attain a subepithelial position, they tend to be broken up into smaller groups of cells which then undergo further differentiation into ova and follicle cells.

The thickening of the epithelium and the extension of crypts, tubes, and solid cords into the underlying stroma we believe may be partially explained on the basis of the rapid remarkable volume changes occurring with the development and regression of vesicular follicles and corpora lutea and corresponding differences in the surface extent to be covered by the epithelium.

Further demonstrations of the origin of ova and follicle cells from the vitally stained germinal epithelium were found in the form of completely differentiated primordial follicles, two or three cells deep to the epithelial surface in which one or several ink particles were demonstrable either in one or more of the follicle cells (fig. 8) or the cytoplasm of the ovum (fig. 9). In more deeply lying follicles ink particles were found in some of the low cuboidal follicle cells. In the cases illustrated there were usually several small inclusions at different focal levels, possibly only one at the photographic level.

More advanced stages of follicles in which carbon particles were found either in follicle cells or ova were rare. However, a few examples of nearly mature graafian follicles were found with carbon inclusions in the ovum, one of which was the mitotic spindle of the first maturation division (fig. 12).

In many ovaries, which had been removed a long time after the introduction of ink, numerous anovular follicles were found, in the majority of which carbon particles could be found. These appeared as sharply outlined rings of follicular cells which were circularly arranged about a central area which sometimes contained cell debris. No vesicular anovular follicles were found. The cell debris was thought to be the result of degeneration of an ovum. Those masses showing no evidence of a central ovum were doubtless primarily anovular (fig. 10).

In ovaries removed after an interval of 30 days or more, sharply delimited nests of degenerate cells or debris, including possibly one or more macrophages, were frequently found. The cytoplasm of both the degenerating cells and the macrophages usually contained several ink particles. These masses were considered to represent young follicles which had undergone degeneration soon after they had differentiated from the epithelium (fig. 16). In a few cases small masses of interstitial cells surrounding a central macrophage contained ink inclusions. Presumably the ink was phagocytized by the macrophages and interstitial cells following degeneration of the follicle cells originally containing it.

Although corpora lutea were carefully examined in an effort to find ink incusions, none were found. It is of course possible that no vitally stained follicles ever reached ovulation stage, but more probably that dye inclusions were so dispersed during the hypertrophy and hyperplasia of granulosa cells that we failed to find them.

SUMMARY

Study of ovaries removed after varying periods of time following selective intravital staining with India ink of the germinal epithelium revealed the presence of ink inclusions in all stages of ovogenesis and follicular development, which inclusions could only have been acquired while these cells were part of the germinal epithelium.

Ova and follicle cells were found to differentiate in situ in the germinal epithelium or in extensions from the surface in the form of either epithelial invaginations or long tubes or cords.

Ova with cytoplasmic ink inclusions were seen in various stages of growth and maturation, including one oocyte in the metaphase of the first maturation division.

No evidence was found of ovogenesis by epithelial mitosis. It is possible that this mechanism was suppressed by the presence of the inclusions.

Ink particles were also found in some cells of anovular follicles and in circumscribed nests of degenerating cells or interstitial cells. The inclusions found in the latter case were thought to have been phagocytized after degeneration of follicle cells which had originally contained them.

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PLATES

EXPLANATION OF PLATES

All material was stained with Azan Mallory technique except that in figures 3, 8, and 9 which was stained with Delafield's hematoxylin and azure II eosin.

PLATE 1

EXPLANATION OF FIGURES

1 Germinal epithelium of the ovary 24 hours after injection of India ink into the periovarian space, showing perinuclear ink inclusions in practically all cells of the epithelium. × 844.

2 A large clear cell (arrow) with rather vesicular nucleus and a cytoplasmic ink inclusion, the first stage in ovogenesis, is shown in the germinal epithelium of a rat ovary 49 days after the injection of India ink. To the left is another potential ovum with superficial flattened follicle cells with inclusions. × 844.

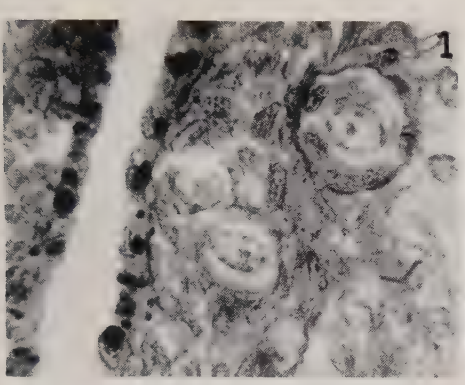
3 Another potential ovum slightly beneath the general level of the germinal epithelium containing three ink granules in its cytoplasm. This and the following figure were from the ovary of a rat 21 days after injection of India ink. × 844.

4 An invagination from the germinal epithelium of the ovary at the base of which is seen another potential ovum with clearly stained cytoplasm containing ink granules and a large, vesicular nucleus. From the same animal as figure 3. × 844.

5 Thickened, wrinkled germinal epithelium with villous-like projections from the surface. From the base of the intervening crypts two masses of ink-laden cells are apparently being cut off from the surface. In the center of one of these is a large pale staining cell, a potential ovum with two large ink particles in the plane of section. Taken from a rat ovary 131 days following the injection in India ink. × 844.

6 Thickened wrinkled germinal epithelium similar to the preceding, showing a deep tube-like epithelial invagination at the end of which is a sharply circumscribed mass of cells. Ink granules are seen in all epithelial structures. Beginning follicular differentiation in the circumscribed mass is apparent. Taken from a rat ovary given the same treatment as the preceding case. × 844.

7 Showing a solid cord of pale staining epithelial, ink-laden cells in continuity with the thickened germinal epithelium of a rat ovary 40 days after injection of India ink. × 844.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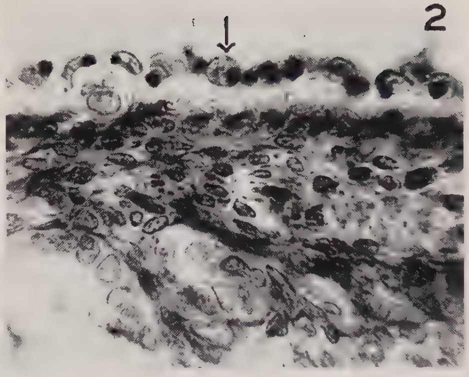
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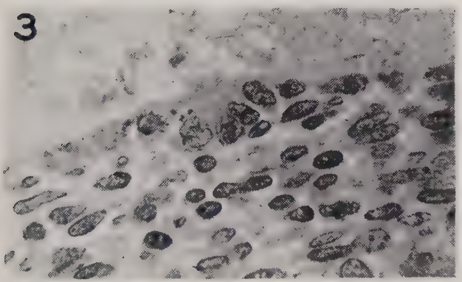
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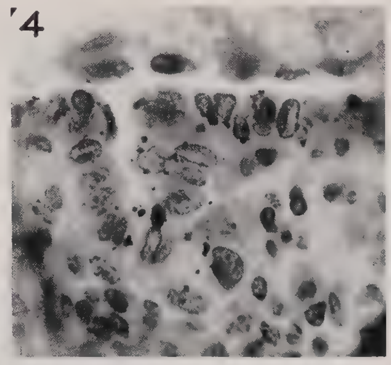
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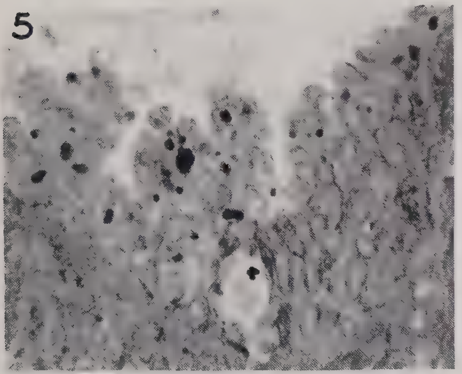
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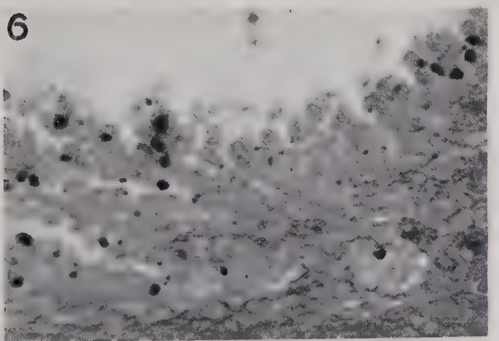
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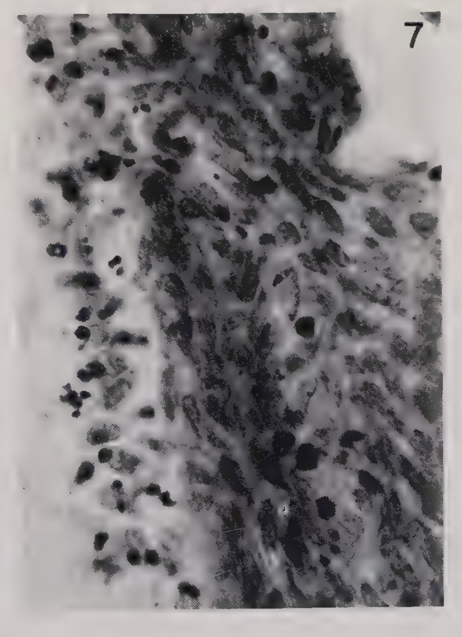
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PLATE 2

EXPLANATION OF FIGURES

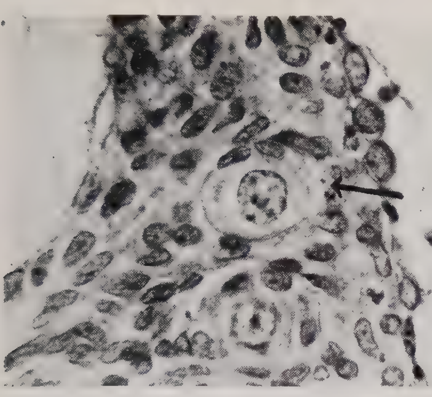
8 This ovary was removed from a rat 24 hours after injection of India ink. Ink inclusions are seen (arrow) in a cell of a primary follicle. × 844.

9 Showing a number of young follicles with an ink inclusion in the cytoplasm of the ovum of one. From the ovary of a rat 22 days after injection of India ink. × 600.

10 A sharply circumscribed nest of follicle cells, some of which contain ink inclusions but with no evidence of a central ovum (anovular follicle). From the ovary of a rat 49 days after injection of India ink. × 844.

11 Circumscribed masses of follicle (?) cells showing fatty degeneration and containing ink inclusions in the ovary of a rat 131 days after injection of India ink. × 600.

12 Ovum with the spindle of the first maturation division, in a nearly mature graafian follicle. Two large ink inclusions are seen near the center of the cell. The mass near the pole opposite the spindle is not a carbon inclusion but a cytoplasmic inclusion common to ova at this stage of development. From the ovary of a rat 21 days following the injection of India ink. × 844.



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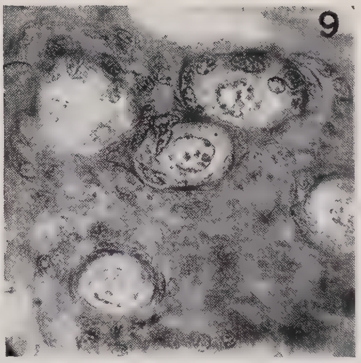

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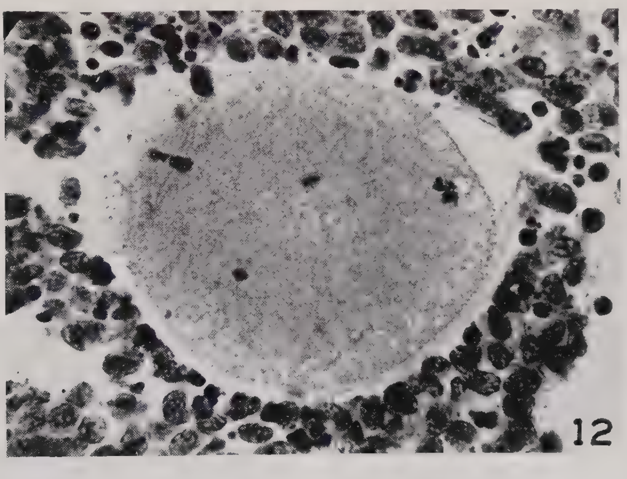

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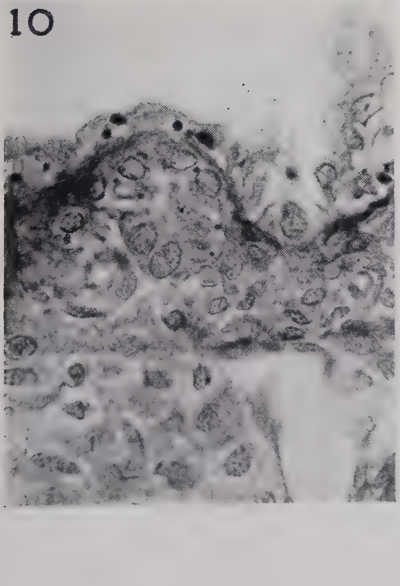

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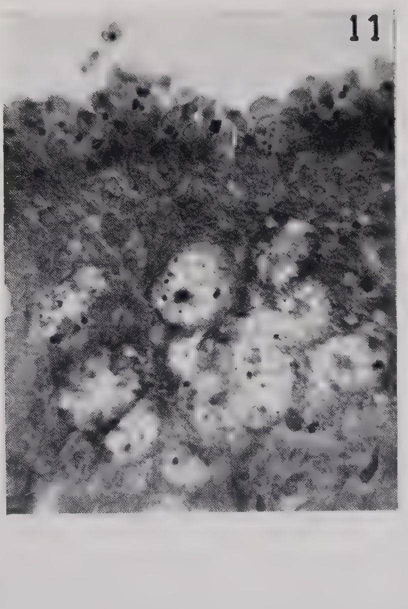

11

THE CAVERNOUS SINUS IN A MACACUS RHESUS MONKEY

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Department of Anatomy, University of Wisconsin, Madison

TWO FIGURES

The usual description of the anatomy of the cavernous sinus is that of a meshwork of fine trabeculae through which certain vessels and nerves pass. Between these strands run endothelial-lined venous spaces which communicate with each other. Morris ('33) states "Its (the cavernous sinus's) lateral wall contains the oculomotor and trochlear nerves, and the ophthalmic divisions of the trigeminus. . . . The internal carotid artery and the abducens nerve are contained in the thinner medial wall of the sinus." Communicating anterior and posterior intercavernous sinuses are mentioned. Sabotta-McMurrich ('14) shows the oculomotor, trochlear and abducens nerves, and the ophthalmic and maxillary divisions of the trigeminus in the lateral wall of the sinus. The internal carotid artery occupies a more central position within the sinus. It is surrounded by fine trabeculae and is clearly not in the medial wall. Poirier and Charpy (1899–1904) and Cunningham ('37) list the nerves from above downward in the human as oculomotor, trochlear, ophthalmic division of the Vth, maxillary division of the Vth. The abducens is stated as occupying a more central position in the sinus. Deaver ('01) shows five separate large channels rather than a network. He places the order of nerves from above downward as oculomotor, trochlear, ophthalmic division of the Vth in the lateral wall and the abducens in the medial wall. He does not include the maxillary division of the Vth in the list.

While studying a series of sections of the orbit of the monkey, which included the region of the anterior half of the cavernous sinus, it was noted that the arrangement of nerves and vessels did not conform to the above description in certain aspects. It was with the idea of describing the arrangement as found in the monkey that the following analysis was made.

A large endothelial-lined space (labelled Cavernous Sinus in figure 1) is seen lying between the internal carotid artery and the lateral surface of the sphenoid bone. This space contains blood cells and appears to be a main channel of the cavernous sinus. Its medial wall is also the periosteum of the sphenoid bone. Its lateral wall consists of a more complex arrangement of dura. Immediately lateral to the sinus strands of dura form a loose meshwork containing the internal carotid artery and a few small endothelial-lined spaces. Lateral to this area the dura is much more compact and contains the trochlear, oculomotor, ophthalmic division of the trigeminus and the abducens. These nerves are so tightly packed together that no space can be seen between them and their sheaths with the one exception of the oculomotor which is separated from the dura by a rather wide space. The ophthalmic division of the Vth nerve has already separated into frontal, nasociliary and lacrimal branches. According to the usual description of both human (Morris, '33) and rhesus monkey (Hartman and Straus, '33) this branching takes place just before the ophthalmic division passes through the superior orbital fissure. The maxillary division of the Vth is seen in the lower left corner of the figure, just before it enters the foramen rotundum. It is surrounded by dura but is outside the cavernous sinus proper. The figure shows the two cavernous sinuses communicating across the midline.

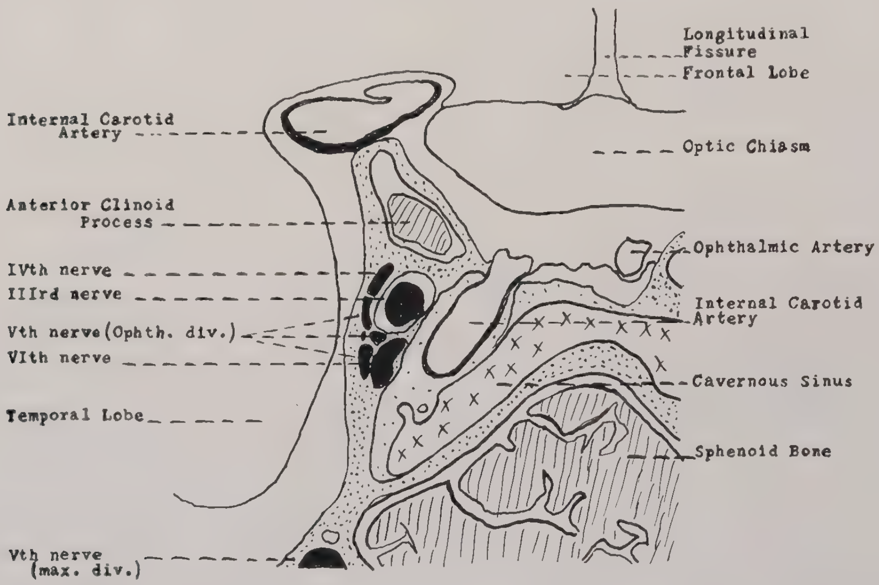
Longitudinal Fissure
Frontal Lobe
Internal Carotid Artery
Optic Chiasm
Anterior Clinoid Process
Ophthalmic Artery
IVth nerve
IIIrd nerve
Internal Carotid Artery
Vth nerve (Ophth. div.)
VIth nerve
Cavernous Sinus
Temporal Lobe
Sphenoid Bone
Vth nerve (max. div.)

Fig. 1 Tracing of section through the region of the cavernous sinus in a Macacus rhesus monkey.

COMMENT

The differences between the usual description of the cavernous sinus and that obtained from sections of the monkey may be stated as follows:

(1) The cavernous sinus in the section studied is more of an entity or main venous channel than is usually described. (2) The abducens nerve is seen just inferior to the oculomotor, in close proximity to the ophthalmic branch of the trigeminus, and not in the medial wall of the cavernous sinus. (3) The maxillary division of the trigeminus does not pass through either wall of the sinus. (4) The medial wall of the cavernous sinus is clearly the periosteum covering the sphenoid bone.

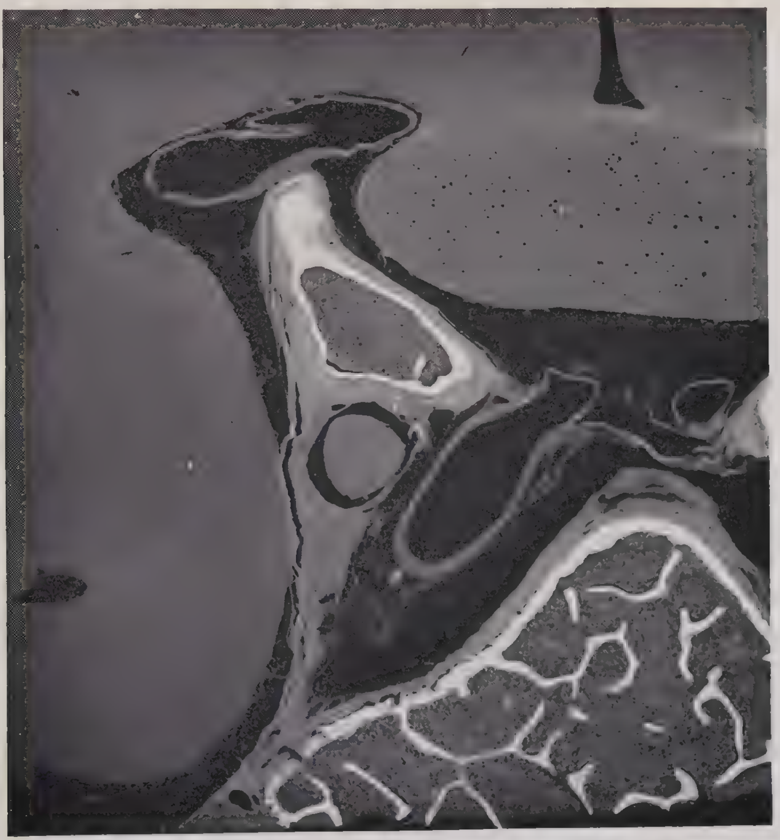

Fig. 2 Section throgh cavernous sinus of monkey.

This dural wall is compact as far forward as the sinus continues and does not contain any large vessels or nerves. If the splitting of the dura which is seen in the lower left corner of the figure is considered as forming the lateral and medial walls of the sinus, then all the vessels and nerves named above as passing through the sinus really lie in its lateral wall.

SUMMARY

1. The cavernous sinus in a Macacus rhesus monkey consists of a venous channel which appears to be less broken up into "caverns" than is usually considered to be the case in man.

2. The lateral dural wall contains two structures usually described as passing through the medial wall of the cavernous sinus, namely the internal carotid artery and the abducens nerve. The compact medial wall forms the periosteum of the sphenoid bone.

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A FIVE-CHAMBERED HUMAN HEART

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TWO FIGURES

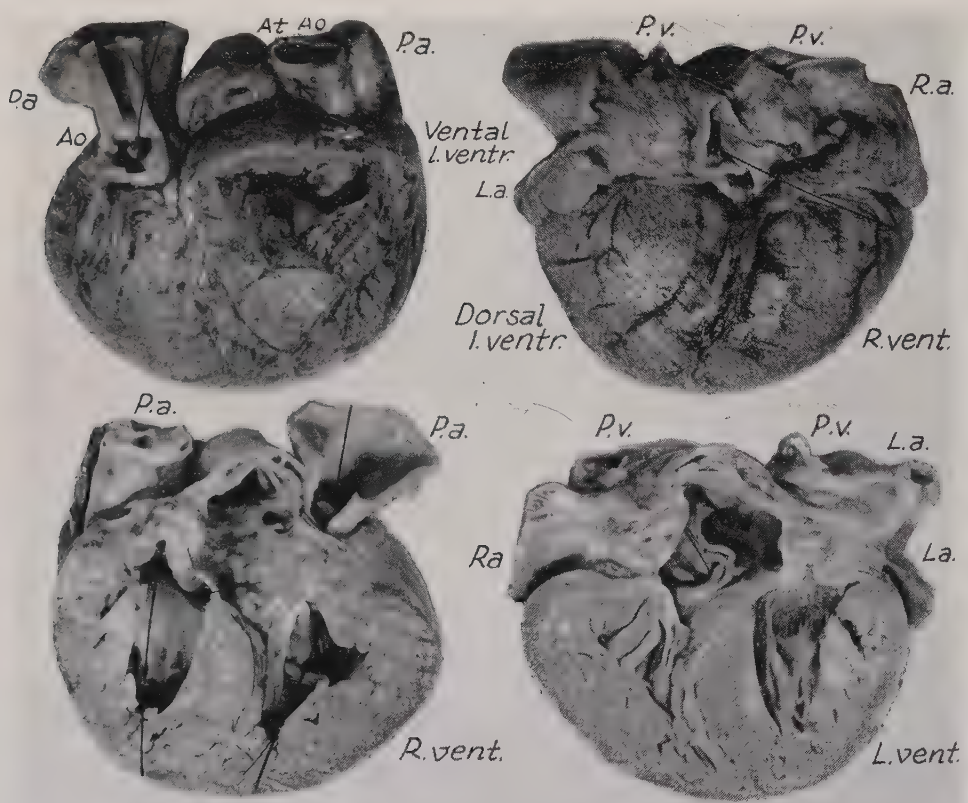
An anomalous heart in the museum of the Department of Anatomy is of such unusual construction that it warrants description. The heart was received by Dr. George Herrmann from Dr. A. L. T. Williams of Vandalia, Illinois, who performed the autopsy in 1933. His letter described its source as a two-headed human monster who was born alive and died shortly thereafter.

The heart measures 44 mm. broad, 42 mm. tall, and 25 mm. deep. It weighs 23 gm., which would be normal weight in an infant. Viewed ventrally, two pairs of large arterial trunks are separated by about 15 mm. The members of each pair are closely fused but do not communicate. Any ductus arteriosus, if present, must have been farther out. A central auricle comes between the pairs. Seen from the dorsal side the atrium shows a large central opening for the vena cavae and a broad opening for the coronary sinus below and to the right. The atrium extends across the top and shows two left and one right auricular appendage. On the roof of the atrium are two widely separated pulmonary venous openings. These reach right and left atria respectively.

The trunks emerging most ventrally are aortae. Each immediately above its valves gives rise to a pair of coronary arteries. There is an anastomosis across the coronary sulcus ventrally and another at the apex of the left ventricle. The dorsal pair also anastomose across the coronary sulcus but not elsewhere. The coronary sinus is well developed on both sides of its point of discharge but particularly to the right.

The pulmonary trunks show several peculiarities. The right pulmonary trunk flares like a trumpet without any arteries leaving it within the area which is visible. The left pulmonary trunk is somewhat smaller but nearly as large as the corresponding aorta. It is capped, however, by a diverticulum to the right and two pulmonary arteries to the left.

Internally the right ventricle is single but the left is double, being divided by an incomplete septum into dorsal and ventral chambers. Each pair of arterial trunks forms a spiral such that the ventrally placed aorta passes to the right of the corresponding pulmonary and reaches the dorsal left ventricle and dorsal part of the right ventricle. The pulmonary trunk arises from the ventral half ventricle which, on the left side, is smaller than the dorsal chamber.

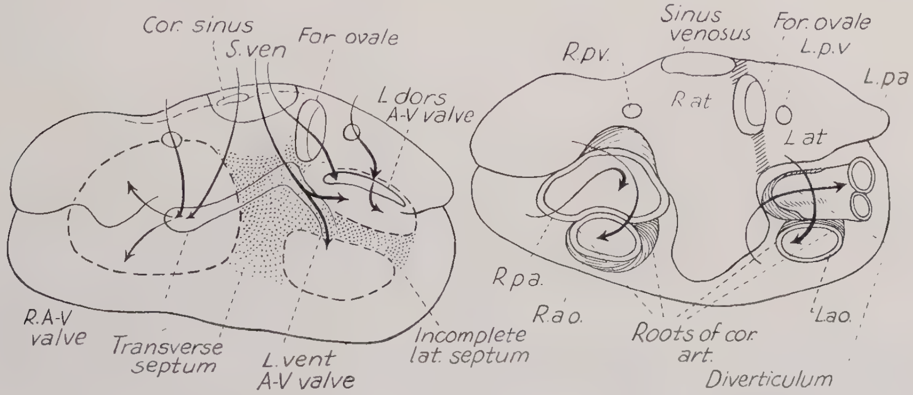


Five chambered human heart

Figure 1

The centrally placed right atrium receives the venae cavae. Below and to the right a large crescentic opening drains the coronary sinus. To the left of the caval opening is a single-leaf crescent valve to the foramen ovale, complete from its attached dorsal margin to its free ventral edge. The opening is large. Capacity of the left atrium and auricles about equals the right. The right atrio-ventricular valve is well formed and dorsal in position. The valve on the left side is more complex, being drawn out over a slit-like but extensive opening which allows blood from the right auricle to reach the three ventricles over the T-shaped interventricular septa.

This heart undoubtedly functioned reasonably well for a long time. Both the arterial and the atrioventricular valves were competent to


Cor. sinus
S. ven
For. ovale
L. dors A-V valve
R.A-V valve
Transverse septum
L. vent A-V valve
Incomplete lat. septum
Sinus venosus
For. ovale
L.p.v
R.p.v.
L.pa
R at
L at
R pa.
R.a o.
Roots of cor. art.
L.ao.
Diverticulum

Atrio-ventricular flow Ventricular aortic flow

Diagram of the heart in dorsal view

Figure 2

hold blood under pressure though the pressure relations in lung and trunk must have been abnormal. A certain amount of doubling of thoracic viscera must have been present. The time of origin of the anomaly was probably coincident with the axial duplication and most studies place that at the time of primitive streak and head process formation.

NOTE ON A FASCIA UNDERNEATH THE PECTORALIS MAJOR MUSCLE

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TWO FIGURES

The purpose of this communication is to call attention to the fact that attached to the under surface of the pectoralis major muscle is a fascia which possibly has not been described before.

From figure 1 it will be seen that this fascia extends along the entire under surface of the pectoralis major muscle except where this muscle has its origin and insertion. At the site of origin of the inferior and medial fibers of the muscle, the fascia blends with the deep pectoral fascia. Superiorly it fuses with the infra-clavicular fascia as this fascia continues as the inferior investment of the subclavius muscle. At the site of the fossa ovalis infraclavicularis (Eisler, '12), it helps to form the inferior and deeper portions of this ring which allows the passage of the nerves and blood vessels supplying the pectoralis major muscle. Here it joins the coraco clavicular fascia. Lateral to this fossa it passes over but is separated not only from the coracoid process and upper portions of the coraco brachialis muscle but also from the biceps tendons; it finally fuses with the deep fascia again underneath the insertion of the pectoralis major tendon on the humerus. Laterally, the fascia joins the thin perimysium of the pectoralis major muscle to become thicker superiorly where it blends with the axillary fascia. The fascia is very loosely connected to the delicate investment fascia over the anterior aspect of the pectoralis minor muscle. The thickest portions of the fascia are where it supports the vessels and nerves after they have left the infraclavicular fossa ovalis; the thinnest portions are near the inferior and medial origin of the pectoralis major muscle.

From figure 2 it will be seen that the fascia is a relatively thick membrane, separated from the under surface of the pectoralis major muscle by a layer of fat, and lying close to the anterior aspect of the pectoralis minor muscle from which it is separated by a thin, delicate membrane of collagenous fibers. The fascia is of approximate uniform thickness except where it is joined by connective tissue septa which usually run parallel and anterior to it. It is composed of fibroblasts and collagenous fibers and of a substantial contribution of elastic tissue elements. The elastic tissue is in several layers of thin strands, frequently fragmented, and connected with the fine and thinner threads of the same tissue appearing in the delicate connective septa which traverse the fatty subpectoral layer to reach the deep surface of the pectoralis major muscle and its investing fascia.

In contrast to the large amount of elastic tissue in the fascia, fibroblasts are relatively few. Collagen fibers, however, are in much greater

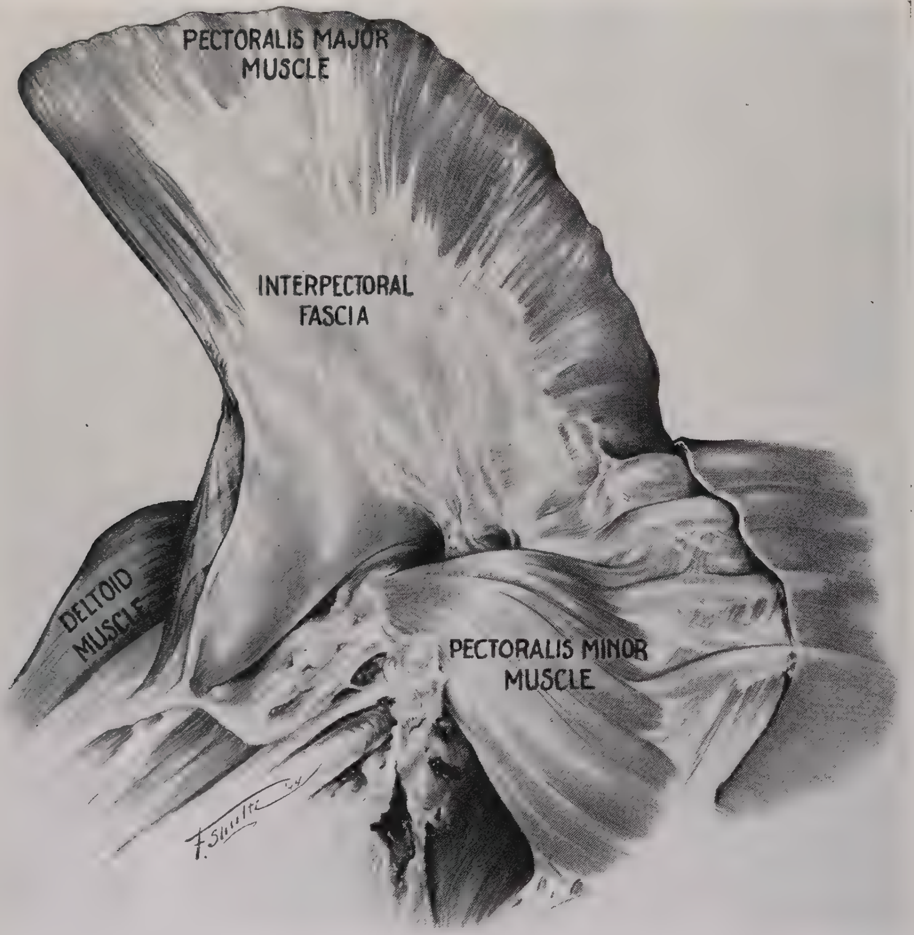
PECTORALIS MAJOR MUSCLE
INTERPECTORAL FASCIA
DELTOID MUSCLE
PECTORALIS MINOR MUSCLE
F. Schulte '24

Fig. 1 Drawing of a dissection of the right upper chest on a cadaver. The pectoralis major muscle has been cut at its origin in the chest, and has been reflected upward and outward to show how the fatty areolar tissue on the under side of the muscle is limited by the interpectoral fascia.

number, and they exceed, on the average, the elastic tissue in amount by only a small margin. In the fatty areolar tissue between this fascia and the under surface of the pectoralis major muscle are seen sections of blood vessels and nerves. The fascia herewith described should not be confused with the investment fascia situated directly on the underneath surface of the pectoralis major muscle. A fatty layer containing the blood vessels and nerves to the pectoralis major muscle separates these two layers.

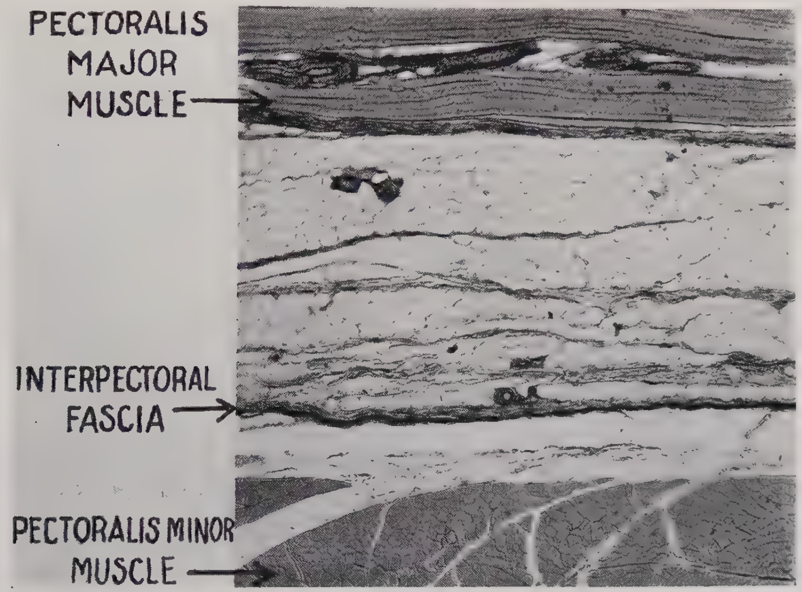

Fig. 2 Photomicrograph of a section of a block of tissue obtained at necropsy from a woman of 35 years with an average build who died from a rapidly spreading melanosarcoma. The tissue was taken where the direction of fibers of the pectoralis major and minor muscles are at right angles; the block included parts of these muscles and all intervening tissue. It will be seen that the interpectoral fascia with its black staining elastic tissue is near the pectoralis minor muscle, yet separated from it by a delicate investment fascia of that muscle. Immediately underneath the pectoralis major muscle is its relatively heavy investment fascia, and between this fascia and the interpectoral fascia is a comparatively thick layer of fatty areolar tissue having blood vessels and thin partitioning septa. Celloidin imbedding, Weigert's elastic tissue stain with eosin counterstain. × 40.

It is not believed that the fascia described above is the deep pectoral fascia detailed by Eisler ('12). A free translation of his description of the deep pectoral fascia is as follows:

On its under surface, the pectoralis major muscle has a strong perimysium externa, which, through loose, partly filled connective tissue masses, is in connection with the deeper fascia structure and the rib perichondrium but independent of the pectoralis major muscle. Only toward the axillary border is the perimysium and the deep fascia melted together. In the loose tissue in between, the anterior thoracic nerves and vessels run to the pectoralis major muscle. Only after removal of the loose masses does the real fascia pectoralis profunda appear. It extends from the clavicle and the medial ends of the ribs and intercostal spaces to the floor of the axilla and to the coracobrachialis muscle, surrounding the subclavius and pectoralis minor muscles . . . .

Examination of figure 78, page 505, in Eisler's ('12) article, shows the deep pectoral fascia placed directly upon the pectoralis minor muscle, indicating, as his text mentions, an envelopment of that muscle and not simply the application of a contiguous membrane. It is not clear from his description whether Eisler ('12) identified this new fascia or whether he included it in his concept of the deep pectoral fascia. The point is that this new fascia, to which the name fascia inter-pectoralis musculorum is given because of its situation between the pectoralis major and minor muscles, is more closely identified with the pectoralis major muscle than with the pectoralis minor. Its main function is to serve as a deep limiting membrane for the fatty areolar layer immediately underneath the pectoralis major muscle, this fatty layer serving as a vehicle for the transmission of the chief nerve and blood supply to this muscle. In addition, the fascia facilitates the movement of the pectoralis major over the pectoralis minor muscle.

The fundamental relationship of the interpectoral fascia can be readily demonstrated at the operating table when a radical amputation of the breast is carried out. In one of the steps in this operation the fingers are inserted between the pectoralis major and pectoralis minor muscles, and the natural plane of separation is found to be between the interpectoral fascia and the pectoralis minor muscle, and not between the interpectoral fascia and the pectoralis major muscle.

The interpectoral fascia was present in every one of the forty adult bodies used for the routine dissection by the class. It was best seen when the layer of fat superficial to it was well developed; it was correspondingly smaller and thinner when the fatty layer was less pronounced. Apparently this fascia has not been recognized as an entity before; it is not described in the current standard text-books of anatomy, nor in the special treatises on fascias by either Gallaudet ('31), or Singer ('35). At no time did it approach in size and consistency the heavier serrato-costal fascia recently reported.

Through the cooperation of Dr. Adolph Schultz it was possible to examine the preserved bodies of several of the higher apes in order to see whether the interpectoral fascia had any representation in these animals. In a female gorilla about 2 years old it was found that the fascia was well developed superiorly, arching toward the fossa ovalis infraclavicularis; the anterior thoracic nerves and blood vessels were superficial to the fascia. In a male chimpanzee, about 35 years old, the interpectoral fascia was again well represented; and in an adult female Macacus rhesus monkey the fascia was again seen. In this last instance the fatty layer superficial to the interpectoral fascia was unusually large. To what extent this deposit of fat as well as the extent of the interpectoral fascia represents an adaptation to the swinging motion at the shoulders is a matter for speculation.

SUMMARY

Between the pectoralis major and minor muscles of the human body is a fascia placed nearer to the pectoralis minor muscle, but anatomically more closely associated with the pectoralis major muscle from which it is separated by a layer of fat which transmits the nerves and vessels supplying most of the pectoralis major muscle. This fascia, which contains a large amount of elastic tissue, can be stripped easily from the pectoralis minor muscle. It is not identical with the deep pectoral fascia, and the name of fascia interpectoralis musculorum is suggested for it. It was found also in the gorilla, chimpanzee and Macacus rhesus monkey.

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EFFECT OF COLCHICINE ON MITOSIS IN THE NEURAL TUBE OF THE FORTY-EIGHT HOUR CHICK EMBRYO[1]

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INTRODUCTION

One of the effects of colchicine upon mitotic nuclei is to produce metaphase block. At adequate concentrations and as time goes on more and more nuclei are thus affected, so that in counts the number of mitotic nuclei appears to increase. This increase was first interpreted by many to mean that mitosis had been stimulated. It was then realized that if all mitosis in progress at a given time is arrested at the metaphase an increase might be the result simply of an accumulation of nuclei in this phase (Ludford, '36).

The latter interpretation is now generally accepted, but even recently certain results with colchicine on animal material have been attributed to stimulation. This is the case, for example, in the chick in which Paff ('39) found that one result of treating 48-hour chick embryos with colchicine was to produce local regions of overgrowth in the neural tube. These he attributed to mitotic stimulation. It seems desirable, therefore, to determine in a quantitative way, if possible, whether or not mitosis is stimulated by colchicine. This is especially important in view of the frequency with which colchicine is being used to arrest mitosis as an experimental method.

MATERIAL AND METHODS

The window technic of introducing substances to the embryo chick leaves much to be desired in speed and in the assurance that the embryo is uniformly surrounded by the substances introduced. Although we tried this method and various modifications of it in preliminary experiments, it was soon abandoned in favor of the method described below which we found to be simpler and to give more uniform results.

Eggs were incubated slightly over 48 hours. Each egg was then opened, and most of the contained albumen was poured into a 100-cc. beaker containing 10 cc. of a solution of colchicine in a warmed, physi-

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[1] The senior author is indebted to the Division of Natural Sciences, Vanderbilt University, for financial aid in this work.

ological salt solution. The albumen and colchicine solution were then mixed by agitation and the intact yolk and remaining albumen were added. A strip of gauze was wrapped around each beaker so as to project over the rim, and a glass plate was rested on the edges of the cloth as a cover. Each beaker with contents was placed in an incubator where it remained for some measured time.

Embryos so treated and controls were removed from the yolks in the usual way. They were fixed in warmed B-15 fluid, dehydrated very gradually, cleared and imbedded. Sections were prepared and stained with iron alum hematoxylin. Counts were made of the number of nuclei in each of the phases of mitosis and of the number of resting nuclei. A modified Howard micrometer disc ruled into nine squares was used in counting. Counts were recorded for each micrometer area, and these were combined at random until a total number of nuclei near 2000 was reached. These totals were then used as samples in determining the standard deviations and were further combined for presentation. Special care was taken to make counts here and there over the neural tube, since it is known (Derrick, '37) that the mitotic index varies widely in different regions.

The method described above of maintaining embryos during the experimental period must furnish conditions which are essentially normal well beyond the duration of the experiment (3 hours), for controls (10 cc. of salt solution plus albumen) were normally developed after 24 hours. Incidentally several embryos were carried successfully well beyond this period, even up to 2 and 3 days.

Concentrations of colchicine ranging from 0.0078 mg., to 1.0 mg., per embryo were tried in preliminary experiments. At all of these concentrations effects on mitosis appeared as early as 20 to 30 minutes after treatment had begun. Since it was not desired to carry these experiments beyond a few hours, that dose was selected (1.0 mg.) which produced complete block of most nuclei within 3 hours. All of the data here recorded are based upon embryos treated with this amount for periods of time ranging from 40 minutes to a maximum of 3 hours.

OBSERVATIONS AND DISCUSSION

The results of our experiments are shown in table 1. Considering the percentage of each phase of mitosis referred to the total number of nuclei counted, the data show clearly that there is no increase in the percentage of any phase except metaphase. If colchicine acted as a stimulant there would have to be an increase in all phases of mitosis. The increase in metaphase can be entirely accounted for in terms of accumulation of arrested cells. Thus, anaphases and telophases have almost completely disappeared after 40 minutes, while at this time the per cent of prophases is still almost unchanged, indicating that cells are still coming through prophase to metaphase at the normal rate. The increase in metaphase during these 40 minutes, from 2.4% to 10.6%, represents the nuclei which during this time have entered metaphase.

TABLE 1

Effect of 1.0 mg., of colchicine on mitosis in the neural tube of the 48-hour chick. The percentage of each phase of mitosis is based on the total number of nuclei counted. In some cases only prophases were counted in certain samples, so that the sum of the percentages of each phase may not always equal the mitotic index.

TIME TREATED	NUMBER OF NUCLEI COUNTED	NUMBER OF MITOTIC NUCLEI	MITOTIC INDEX	STANDARD DEVIATION	PER CENT PROPHASE	PER CENT METAPHASE	PER CENT ANAPHASE	PER CENT TELOPHASE
Control	6363	598	9.3	± 1.1	2.8	2.4	0.2	4.6
40 minutes	14534	1989	13.6	± 1.1	2.4	10.6	0.0	0.3
1 hour	3928	627	15.9	....	3.0	8.4	0.0	0.0
1½ hour	12816	1670	13.0	± 3.8	2.3	9.2	0.0	1.4
2 hours	19074	3240	17.1	± 3.1	0.6	15.8	0.0	0.1
3 hours	19579	3977	20.2	± 4.2	1.1	20.5	0.0	0.0

In view of the results of these experiments the regions of excessive overgrowth in the neural tube described by Paff ('39) and observed in our material also must be attributed to some other cause than colchicine stimulation. Sauer ('35) has shown that in the neural tube epithelium nuclei migrate from the periphery into the germinal layer where they then undergo division, returning then after completing division. Since colchicine arrests the division at metaphase, it may be that the return of resting nuclei is also prevented but their migration into the germinal layer is not. If this be true, nuclei would then tend to accumulate in the germinal layer producing the characteristic swellings or regions of apparent overgrowth.

SUMMARY

1. Chick embryos of 48 hours incubation were treated with 1.0 mg., of colchicine and allowed to continue development for different lengths of time. Counts were then made of the number of resting and mitotic nuclei in the neural tube.

2. The mitotic index rises to a maximum of 20.5% in 3 hours. The percentage of each phase of mitosis, except that of metaphase, eventually declines. The rise in metaphases can be accounted for in terms of the accumulation of nuclei in this phase. It is concluded that colchicine at this concentration acts only in this way and not as a mitotic stimulant.

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EFFECT OF BIOTIN DEFICIENCY ON EMBRYONIC DEVELOPMENT IN THE DOMESTIC FOWL[1]

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FIVE FIGURES

The essential nature of biotin in reproduction in the domestic fowl has been previously reported by Cravens, Sebesta, Halpin and Hart ('42). In a further experiment (Sebesta, et al., '43) the approximate biotin requirements of breeding hens have been established. In the latter experiment all eggs failing to hatch were broken and the embryos examined for gross pathological symptoms and the approximate age at death as recorded in table 1.

MATERIALS AND METHODS

Twenty-four Single Comb White Leghorn pullets were placed in individual laying cages after being reared on the Experiment Station Range. All birds were artificially inseminated weekly with mixed semen from ten Single Comb White Leghorn cockerels. All eggs laid were marked with the hen's number and settings were made weekly. The eggs were candled on the sixth, thirteenth and seventeenth days, at which times the infertile eggs and dead embryos were removed. The time of death was estimated by the stage in development that had been reached.

The basal ration fed the birds had the following composition: sucrose 61, gelatin 5, alcohol-extracted casein 18, salts IV 5, Hegsted et al. '41) soybean oil 5, solubilized liver extract[3] 4, fish oil (3000 I. U. vitamin A, 400 A.O.A.C. units D) 2. Crystalline vitamins[3] were added per kilogram as follows: thiamin 3 mg., riboflavin 5 mg., calcium pantothenate 15 mg., nicotinic acid 100 mg., pyridoxin 6 mg., and choline 2.0 gm. Oyster shell was supplied ad libitum. The birds were divided into six groups of four birds each and were given 0, 30, 60, 90, 120 and 150 micograms of biotin concentrate[3] per kilogram of ration respectively. Groups 1 and 2 contained only three birds inasmuch as one bird from group 1 died early in the experiment and one from group 2 was discarded because many of her eggs were broken when laid. The experimental period was 12 weeks. A few birds were continued for 7 additional weeks because certain embryonic symptoms were noted making additional study desirable.

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[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] The Borden Co. Industrial Fellow.

[3] We are indebted to The Borden Co., Special Products Division for the fish oil; to Wilson Laboratories, Chicago, Ill., for the solubilized liver extract; to Abbott Laboratories, North Chicago, Ill., for the biotin concentrate and to Merck and Co., Rahway, N. J., for the crystalline vitamins.

EXPERIMENTAL RESULTS

Time of embryonic mortality. The percentage of embryonic deaths at various estimated ages is given in figure 1. The results of only four groups are presented — those receiving 0, 30, 60 and 150 micrograms of biotin per kilogram of ration — inasmuch as these groups show quite clearly the effect of different degrees of biotin deficiency on age at death of the embryo. Group 1 was considered as being severely deficient in biotin, group 2 was moderately deficient, group 3 as slightly deficient and group 6 as adequate.

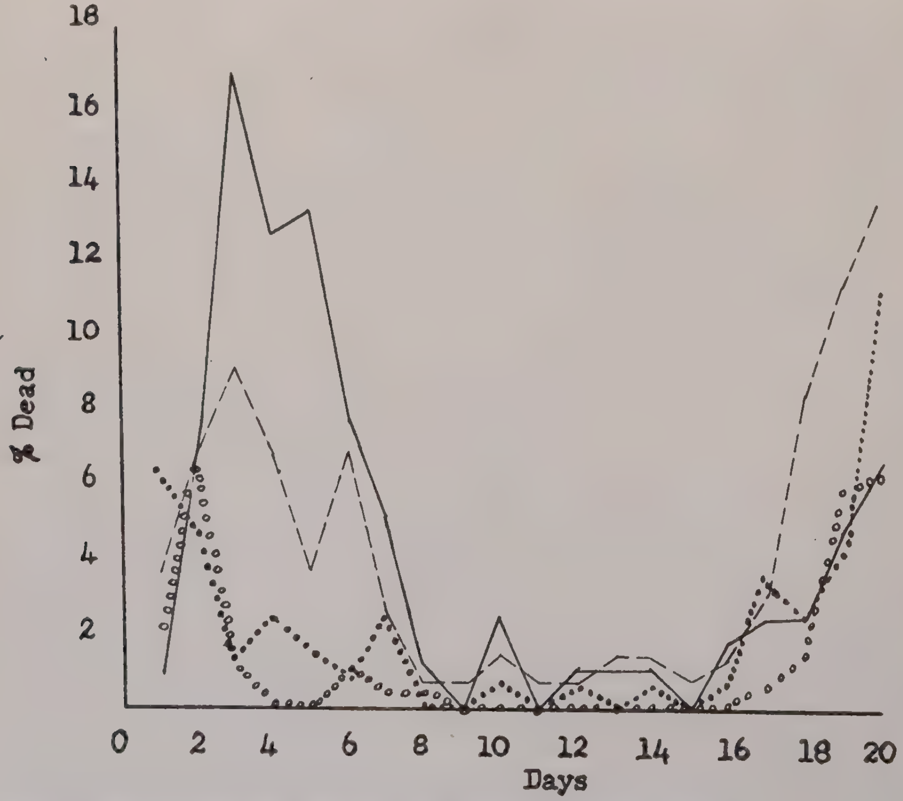


Fig. 1 Solid line indicates basal diet only; broken line basal plus 30 micrograms; dotted line basal plus 60 micrograms; circles basal plus 150 micrograms of biotin per kilogram of ration respectively.

TABLE 1

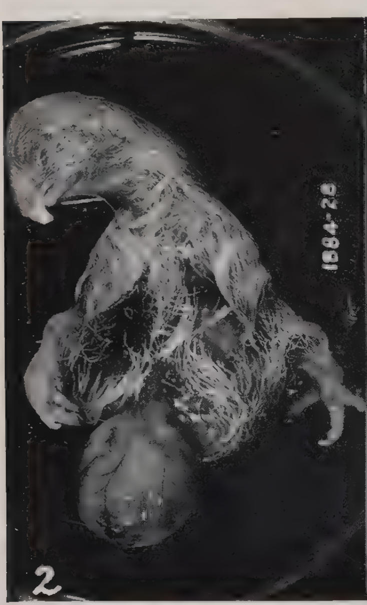
Classification of embryonic abnormalities from hens fed varying amounts of biotin.

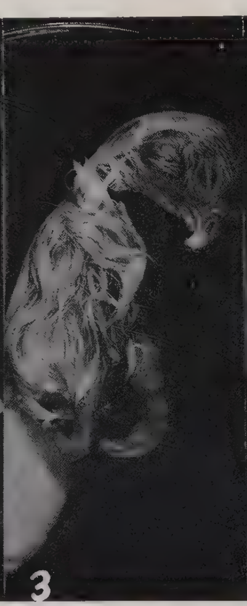
HEN	MICROGRAMS OF BIOTIN PER KILOGRAM RATION	FERTILE EGGS	EMBRYOS DYING		NUMBER OF EMBRYOS SHOWING											
			Before 8 days	8 days and after	Hemorrhage		Down		Edema	Chondrodystrophy	Crooked tibia	Short twisted tarsus	Size		Syndactyly	
					Body	Allantoic	Short	Clubbed					Dwarf	Small		
1881	0	59	49	6		2				2	1	1		1	0	1, 6[1]
1884	0	52	27	22	7	9	3	3	4	10	11	9	5	7	2	0, 1[1]
1885	0	57	32	12	2	5		2				1	2	2		2, 3[1]
Total		168	108	40	9	16	3	5	4	12	12	11	7	10	2	3, 10
1886	30	37	15	17		4	2	2	1	5	7	6	1	4	2	0, 5[1]
1890	30	39	9	20	3	6			2	5	4	5	1	3	8	2, 7[1]
1893	30	57	29	23	3	7	1	1	1	4	3	3	4	5		3, 8[1]
Total		133	53	60	6	17	3	3	4	14	14	14	6	12	10	5, 20
1894	60	46	5	18	1	7	1	1	1					3	1	3, 12[1]
1896	60	21	7													
1898	60	50	12	7		3				1				1		0, 5[1]
1900	60	11	1	7		1	1			1	2	2		2	4	
Total		128	25	32	1	11	2	1	1	2	2	2		6	5	3, 17
1901	90	55	13	6	1	3									1	
1903	90	56	12	8		2								1		
1905	90	41	6	4		2									1	
1916	90	45	8	5		1										
Total		197	39	23	1	8								1	2	
1917	120	22	1	8		3			1							
1918	120	11		2				1			1	1		1		
1923	120	8		1												
1925	120	42	5	7									1	1		
Total		83	6	18		3		1	1		1	1	1	2		
1930	150	48	6	3												0, 3[2]
1936	150	44	3	5		4										
1941	150	59	6	5		1			1							0, 1[2]
1942	150	48	8	14		3	3		1		2			2		0, 3[2]
Total		199	23	27		8	3		2		2			2		0, 7

[1] Birds continued on basal ration for an additional 7 weeks, first figure in column represents number of embryos showing syndactyly, second embryos 8 days and over examined.

[2] Continued on 150-microgram level of biotin.

Gross pathological symptoms. The more common gross pathological symptoms observed are given in table 1. The results of individual hens are presented because of the considerable individual variation in the incidence of any particular abnormal character. In these studies the term chondrodystrophy has been arbitrarily limited to embryos showing a definite parrot beak. The appearance of syndactyly was not observed until the experiment was well under way and for this reason eight birds were fed the basal ration and four were continued on the 150-microgram level of biotin for an additional period of 7 weeks to further observe the incidence of this condition. The results of these additional examinations for syndactyly are given in the last column of table 1. Typical examples of chondrodystrophic and syndactylous embryos found in this study are shown in figures 2–5.





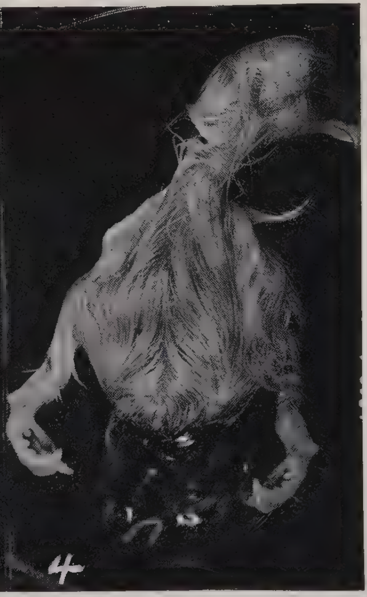


Fig. 2 Ventral view of embryo from biotin deficient hen showing parrot beak, crooked tibia and short twisted tarso-metatarsus. (Estimated age 19 days.)

Fig. 3 Lateral view of embryo from biotin deficient embryo showing parrot beak, crooked tibia and short twisted tarso-metatarsus. Embryo recorded as small. (Estimated age 18 days.)

Fig. 4 Dorsal view of embryo from biotin deficient hen showing parrot beak, crooked tibia, short twisted tarso-metatarsus and syndactyly. (Estimated age 19 days.)

The other pathological symptoms recorded in table 1, namely hemorrhage, retarded or clubbed down, edema and size are not discussed in detail because of the small numbers and the lack of clear-cut evidence that biotin is a factor in their prevention. Embryos exhibiting hemorrhage were divided into those showing hemorrhage in the body and those having hemorrhage in the allantois. In the original data the degree of hemorrhage was estimated but because of the small numbers involved, those of all degrees were summarized together. The most commonly noted edema occurred under the neck or on the sides of the body. The down was listed as being short when its development appeared retarded in relation to the estimated age. Clubbed down most frequently occurred on the abdomen and under the neck. An embryo was termed small when its size appeared definitely reduced in relation to estimated age. Dwarf embryos were obvious as they were extremely small for their estimated age. It should be stated, however, that the average number of abnormal symptoms per embryo decreased as the level of biotin in the diet of the mother hen was increased.

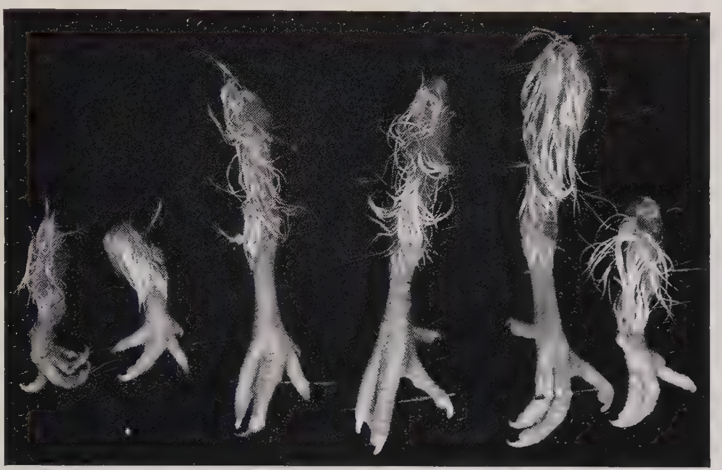


Fig. 5 View of right leg of embryos showing a reduction in length, usually found associated with parrot beaks. The three short legs exhibiting crooked tibia and short twisted tarso-metatarsus were from embryos having parrot beaks. The four legs on the right exhibit syndactyly.

DISCUSSION OF RESULTS

It may be seen from the data in figure 1 that a deficiency of biotin in the incubating egg is associated with a pronounced increase in embryonic mortality during the first week of incubation with a peak about the third day. Relatively few embryos die between the eighth and sixteenth days of development but another peak in embryonic mortality occurs during the last 3 days, especially when the hens were fed small but suboptimal amounts of biotin. These so-called critical periods have been known for some time and a further critical period at about 13 days has been reported in case of a riboflavin deficiency. Lepkovsky, Taylor, Jukes and Almquist ('38); Romanoff and Bauernfeind ('42). The latter authors reported that, in case of a riboflavin deficiency, the time of death seemed to be controlled to a great extent by the degree of depletion of the laying hen. This also seemed to be true in a biotin deficiency although in no instance was there a pronounced increase in embryonic deaths at the second critical period. The transition from live chicks to embryos dying during the first critical period took place within a very short period of time after the breeding hens were placed on the basal diet. The basal group showed a very high incidence of embryonic mortality during the first critical period while groups 2 and 3, which received small but inadequate levels of biotin, showed less mortality during early embryonic development and more during the latter stages or the third critical period.

The increase in early embryonic deaths may not be considered as being due specificially to a lack of biotin. For example, Romanoff and Bauernfeind ('42) reported a very high embryonic mortality at about the fourth day among embryos from hens fed diets markedly deficient in riboflavin. Adamstone ('31) reported similar findings in the case of a vitamin E deficiency. Abnormally high incubator temperatures have also been reported to cause high embryonic mortality during the first critical period. Alsop ('19); Romanoff, Smith and Sullivan ('38). These results with biotin, however, do suggest that this factor plays a very important role in early embryonic development.

The apparent increase in embryonic mortality at the third critical period, probably due to a biotin deficiency, has also been reported to occur among embryos from hens fed diets deficient in vitamin D by Insko and Lyons ('36). The same authors ('37) found that a deficiency of manganese in the diet of the laying hen likewise increased embryonic mortality at the third critical period.

The figures given in table 1 as well as the photographs show that a deficiency of biotin in the diet of the laying hen is one factor responsible for certain abnormalities in the developing embryo. In view of the relatively small numbers involved in this experiment, it is impossible to draw any definite conclusions regarding the effect of biotin on the incidence of hemorrhage, retarded down development and edema in embryonic development of the chick. It is worthy of note, however, that the incidence of the above pathological conditions was highest among embryos from hens fed the basal diet and the 30-microgram level of biotin. Some cases of each abnormality were observed, however, among embryos from all groups. The incidence of embryos recorded as either small or dwarf was much higher in groups 1 and 2 than in groups 3–6.

The most striking symptom observed among embryos from the biotin deficiency groups was chondrodystrophy. Not a single chondrodystrophic embryo, as defined in this paper, was found among sixty-eight embryos originating from birds receiving 90, 120, or 150 micrograms of biotin while out of 132 examined, derived from birds receiving 60 micrograms or less, twenty-eight exhibited chondrodystrophy.

Chondrodystrophy has been reported to occur under many different conditions. Dunn ('23) first reported the disorder but in later studies was unable to find any genetic basis for it. In 1927 he reported that the accumulated evidence "made it appear doubtful whether the condition is influenced in any way by inheritance." This type has been referred to as sporadic inasmuch as a seasonal incidence has been reported by Hutt and Greenwood ('29) and Munro ('32) and others. More recently, however, Lamoreaux ('42) has described a hereditary type and has presented evidence that it is inherited as an autosomal recessive. A nutritional type was reported by Byerly and co-workers ('33) as it was found to be prevented by allowing the parent stock access to direct sunlight and green range. Lyons and Insko ('37) reported a nutritional type which could be prevented by manganese and which they believed to be identical with the type described by Byerly and co-workers ('33).

The so-called chondrodystrophic embryos found in this study were somewhat reduced in size and in a large proportion of the cases the parrot beak condition was associated with severely crooked tibiae and/or a much shortened and twisted tarso-metatarsus. Grossly the chondrodystrophic embryos resemble the type first described by Landauer and Dunn ('26). They differ somewhat from the type attributed to a manganese deficiency by Lyons and Insko ('37). In contrast to the the straight tibia, marked edema and retarded down development encountered in case of a manganese deficiency, the chondrodystrophic embryos showed crooked tibia, very little retardation in development of down or little edema.

In view of the appearance of chondrodystrophy under such varied conditions, it appears likely that more than one factor is involved in preventing the appearance of this disorder. It is suggested that the factors may be either of genetic or nutritional nature or both and are probably closely interrelated.

Syndactyly or extensive webbing between the third and fourth toes was another condition observed in this study. As already mentioned, its occurrence was limited to one or more embryos from ten of fourteen hens in groups 1 through 4. The condition was not observed in any chicks hatched during the experiment. Though the frequency is admittedly low, the incidence is suggestive that syndactyly may, in part, be dependent for its expression on inadequate amounts of biotin in the diet of the mother hen. Also the possibility cannot be overlooked that heredity may be concerned with the expression of this character. Since the females were assigned to the groups at random and the semen used in insemination was pooled, differences due to heredity between groups were assumed to be minimized. The role of heredity in the expression of this character under different nutritional regimens of the mother hen with respect to biotin will be observed in future experiments.

Observations on the inheritance of syndactyly as a morphological character were reported by Davenport ('09), Danforth ('19), ('29) and Jaap ('39). These authors have presented evidence that syndactyly in some breeds is often associated with feathered shanks and brachydactyly and assumed that it was another expression of the gene or genes conditioning leg feathering. As yet the inheritance of syndactyly is not well understood.

The association of syndactyly with certain other characters (irrespective of cause) is noteworthy. Warkany and Nelson ('41), ('42) have observed syndactyly to be associated with various skeletal abnormalities involving the legs, mandibles, ribs and sternum among the offspring from rats maintained on deficient diets and have shown that these symptoms can be prevented by some nutritional factor present in liver. More recently Waters and Bywaters ('43) have reported the complete association of syndactyly with an inherited lethal mutation in the chicken. These observations, together with our own, are suggestive that syndactyly is in some way related to other than normal embryological development, the causes probably being several.

SUMMARY

Two pronounced peaks of embryonic mortality occurred among eggs produced by hens deficient in biotin. One such peak occurred at the first critical period and the other at the third critical period.

Chondrodystrophy in the embryo appeared to be one symptom of biotin deficiency in the diet of the breeding hen.

Syndactyly also appeared among embryos from hens receiving suboptimal amounts of biotin. No cases were observed among embryos from hens receiving adequate biotin.

The relation of certain abnormalities appearing during this experiment to similar conditions observed under different experimental conditions is briefly discussed.

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PHYSIOLOGICAL FACTORS IN HYPOPHYSIAL-GONADAL INTERACTION[1]

II. VITAMINS AND THE FOLLICULAR MECHANISM OF THE RAT

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TWO FIGURES

The concept of hypophysial-gonadal interaction has been somewhat clarified by results obtained from its experimental modification in certain animals. Among the methods adopted in these studies has been that of prolonged illumination, by which the reproductive cycle may be greatly modified. In some mammals, it has been shown that light accelerates the onset of maturity, and alters the gonadal size and weight; this is accompanied by changes in other structures, as reviewed by Bissonnette ('38), Rowan ('38), and others.

General agreement exists concerning the effects of additional illumination on the sexual mechanism. The physiological factors underlying these effects, and the type of reaction necessary to produce them, however, have not been so satisfactorily elucidated by subsequent experimentation. It is apparent from the work of Fiske ('39; '41), and Pomerat ('42), among others, that the cytology of the anterior pituitary is altered in certain animals under continual illumination. The associated changes in gonads and accessories are thus generally attributed to an alteration in the relative amounts of follicle-stimulating and luteinizing hormones.

Is this a unique action of the gonadotrophic hormones, or do other chemical substances play a significant role in these reactions? That these effects are produced by the specific action of gonadotrophic hormones has not been supported by convincing evidence. Indeed, in certain cases these hormones occupy an anomalous position with respect to their action on the gonads and accessories. Greep et al. ('36) have shown that the follicle-stimulating hormone produces tubular development when extracts are injected into normal and hypophysectomized, immature male rats; the interstitial tissue is not affected. Luteinizing hormone-injections stimulate the development of interstitial tissue, without affecting accessory structures. In adult, hypophysectomized male rats, however, the luteinizing hormone also affects the tubules, since sperm formation is gradually resumed after a 15-day period of atrophy (Greep and Fevold, '37).

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[1] A portion of a dissertation presented for the degree of Doctor of Philosophy in Yale University.

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The great advances in our knowledge of the relationship between the hypophysis and gonads are largely attributable to the intensive studies of gonadotrophic-hormone reactions. Attempts to use this hormonal system as we understand it today as the key to this problem, however, have proved disappointing. Other chemical substances with known chemical structure and physical properties also produce definite changes in gonads and accessories.

Outstanding among chemical compounds with significant effects on the sexual mechanism, are vitamins A and E. It will be sufficient to present a résumé of important modifications of gonads and accessories as reported in studies of vitamin deficiency and hypervitaminosis. Detailed reviews of this subject may be found in the excellent accounts of Mason ('39) and Wolbach and Bessey ('42). Vitamin A-deficiency: produces atrophy of the testis and seminiferous tubules in the male (Mason, '30, '33; Wolbach, '37). In the female, prolonged vitamin A-deficiency results in abnormal cornification of the vagina (Evans and Bishop, '22 a,b), keratinization of the uterine epithelium (Wolbach and Howe, '25, '28; Thatcher and Sure, '32), placental degeneration and fetal death (Mason, '35; Mason and Ellison, '35). Vitamin E-deficiency: produces irreparable degeneration of the testis in the male, as described by Mattill and co-workers ('26) and confirmed repeatedly in numerous forms. The striking effect of E-deficiency in the female is resorption of the placenta and fetus and, in severe cases, degeneration of the uterus (Evans and Bishop, '22 b; Evans and Burr, '27; Juhász-Schäffer, '31; and others).

Our knowledge of the action of large amounts of these vitamins on the sexual mechanism is meagre. We know of no specific affects of hypervitaminosis E on the gonads of either male or female (Demole, '39). Hypervitaminosis A: leads to hyperactive spermatogenesis in the guinea pig (Cornil, et al., '39). There is no account of a specific effect produced by hypervitaminosis A in the female. The belief that overdoses of this vitamin produces deleterious effects (Harris and Moore, '29 a, b; Drigalski and Laubmann, '33) has not received convincing support; it is agreed that the oil carrier of the vitamin, or some extraneous material in the preparation, is responsible for these effects (Vedder and Rosenberg, '38; Bessey and Wolbach, '39; Eddy and Dalldorf, '41; and others).

A note of caution must be introduced with respect to the experiments on vitamin deficiency. Total deficiency of a vitamin, implied by the use of the term ''avitaminosis'' does not exist in some of these experiments; this term is often used to describe what is actually a severe case of hypovitaminosis, in which vitamin reserves are present. The reality of reserves of vitamin A in rats has been demonstrated by Davies and Moore ('37). That its utilization or availabilty is controlled by a special mechanism, is also apparent (Davies and Moore, '35); in several days, sufficient vitamin A may be stored to last, theoretically, for a century. However, the reserve falls rapidly when the animals are placed on a diet free of the vitamin, until a basal level is reached; after this, the animal maintains an adequate reserve which may last for at least 3 months. Furthermore, Dan ('32) and Baumann et al. ('34) have demonstrated continued growth in rats when no measurable amounts of vitamin are present. Conversely, several cases of death occurred in rats with large reserves of vitamin A, and in the absence of lesions to the intestinal tract which might have interfered with absorption. Therefore, unless carefully controlled and prolonged periods of vitamin deficiency are the rule, we can not assume that a condition of avitaminosis exists.

The importance of vitamins A and E in the maintenance of sexual structures and in the successful completion of gestation, indicates the need for a more thorough study of the specific part which these vitamins may play in the normal function of the gonads. In studying the effect of prolonged illumination on the follicular apparatus of the rat (Truscott, '44 b), the experimental animals received adequate amounts of these vitamins. Under light stimulation, the number of non-vesicular follicles increased over the normal without a corresponding decline in the number of vesicular follicles. If vitamins A or E play any significant role in producing these changes, it should be possible to alter this picture significantly by furnishing an oversupply of the vitamins to rats while also increasing their visual stimulation.

In planning and carrying out this experiment, it was recognized that overdoses of one vitamin would be present in animals possessing adequate amounts of the other vitamin. Thus if either of the vitamins exerted a relatively non-specific change in follicular development, this would be hidden by the action of the other vitamin. This phenomenon would be expected in accordance with our knowledge of certain functional relationships existing between these vitamins (Moore, '40;

Davies and Moore, '41). If the modifications under these conditions are as significant as was shown in the general hypervitaminosis studies cited above, however, then we may hope to determine whether these vitamins form an important link in the mechanism of interaction between the hypophysis and the gonads.

Sincere thanks are due Prof. J. S. Nicholas for suggestions, advice, and aid throughout the experiments and preparation of the manuscript.

MATERIAL AND METHODS

The injections of vitamin A used in these experiments consisted of 0.35 cc. (approximately 25,000 I.U.) of a vitamin A solution produced by the International Vitamin Corporation, New York, N. Y. The injections of wheat germ oil (E. Lilly Co., Indianapolis) were administered in doses of 0.30 cc., or approximately 3,000 I.U.

Since some of the vitamins, in particular vitamin E, are not well used parenterally (Goetsch and Pappenheimer, '41), it was considered necessary to introduce the solutions directly into the intestine. The animals were anaesthetized with sodium amytal in the relative dosages prescribed by Nicholas and Barron ('32). A ½ inch incision was made in the ventral abdominal wall, slightly to the right of the mid-line; after the muscles were retracted, the small intestine was exposed and the vitamin was injected with a hypodermic needle into the lumen of the duodenum. This injection was administered slowly because of the tendency of the solution to back up toward the pyloric sphincter. After the injection, the muscles were drawn together by sutures, and the skin was fastened with clips.

Immediately following the injection of vitamin E, the rat was exposed to light for 1 hour; the vitamin A-treated rats remained in a dark room for 10 minutes after the vitamin had been injected, and prior to stimulation by light. With the object of providing a relatively constant and localized source of illumination, the following set-up was used: The rat was placed on a slightly inclined board, and the light from Nicholas lamps was concentrated on each eye so that there was no overlapping between the two fields. The distance of the source of light was gauged so that each eye received a maximum of 30-foot candles.

To provide a period of time comparable to that in which maximum ovarian response is obtained in pituitary activation (Fevold and Hisaw, '34), the rats were returned to their cages for 3 days. The ovaries were then cut at 10 μ and stained in Heidenhain's iron hematoxylin, or in Johannsen's modification of this stain; the latter gave good results and is a shorter method. The ovarian follicles were counted and typed according to the scheme outlined by the writer (Truscott, '44 b).

OBSERVATIONS

Normal rats

The detailed study of nonvesicular (types I and II) and vesicular (type III) follicles was reported in our recent publication ('44 b), and will receive but brief consideration in this report. In rats placed under normal laboratory conditions, the total follicle count and the total number of non-vesicular follicles exhibit a steady decline throughout the age groups studied. The number of type III follicles fluctuates from one age group to another, thus producing significant changes in the relative percentage of follicle types present in the ovary at any given time. Comparison of figure 1 with figure 2 will emphasize the fundamental changes in the follicular apparatus during the early de-

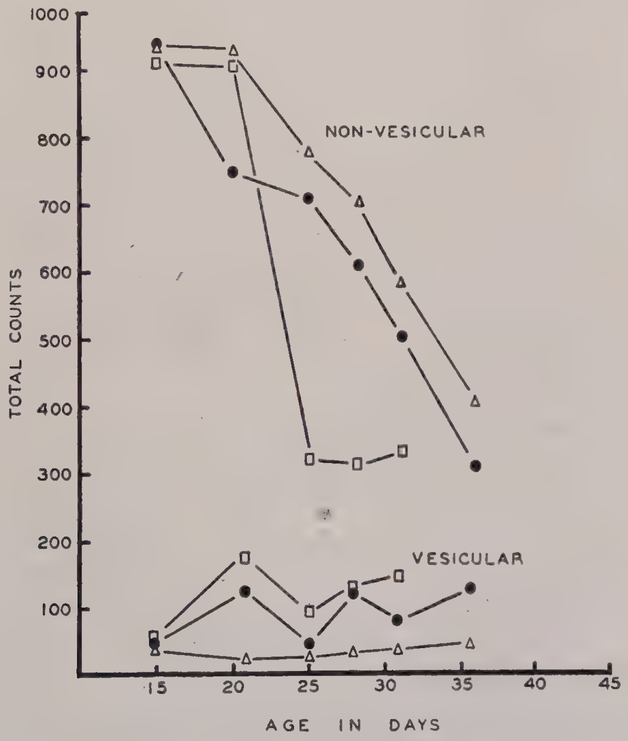

Fig. 1 Graph showing changes in the total number of follicles in normal and experimental animals. Full circle: Normal rats. Full triangle: Vitamin A-Series. Blank square: Vitamin E-Series.

velopment of the normal rat. It will be noted that the number of type III follicles fluctuates around a steadily rising mean as the animal approaches maturity.

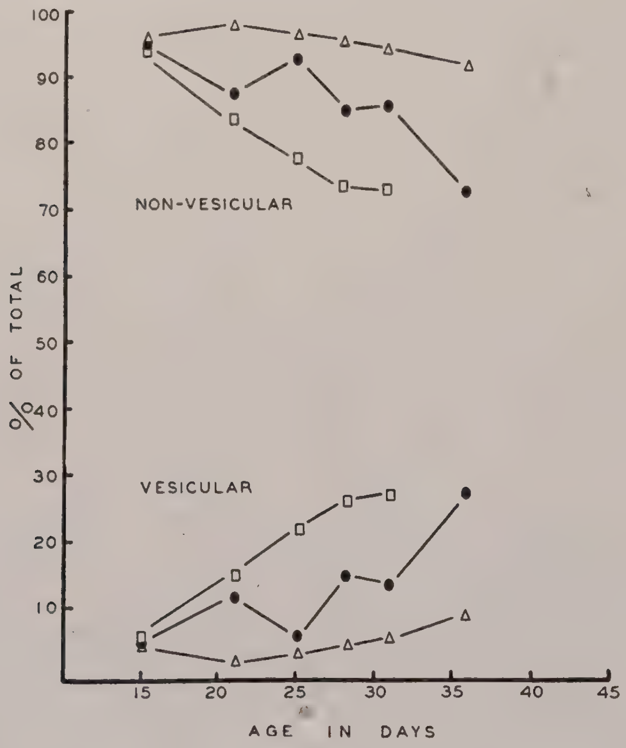

Fig. 2 Graph showing the changes in percentage composition of the total follicle count in normal and experimental animals. For explanation, see legend of figure 1.

Light and vitamin A

The means established for the total follicle count of rats receiving overdoses of vitamin A show a small decline from 984 to 959 between the fifteenth and twenty-first days of postnatal life; this represents a 2.5% difference. On succeeding days, the percentage decrease is sharper being, respectively, 14.5, 9.5, 10.5 and 26.3. It will be noted (table 1) that the higher mean on the twenty-first day, as compared to that of the normal rats, is due to the high level of non-vesicular follicles at this time. Throughout subsequent periods, however, these means are not significantly different from the normal. It may be concluded, therefore, that the total number of follicles is not significantly altered in rats placed under these conditions.

The changes in the means of non-vesicular follicles illustrate the fundamental effects of vitamin A on the follicular mechanism of the rat (fig. 1). Between the fifteenth and twenty-first day there is no significant change in the mean, due partly to the fact that the eyes have just opened at this time and hence visual stimulation is less apt to be effective. The result of the high level of types I and II follicles on the total number at the twenty-first days was noted above. During succeeding stages, the non-vesicular follicles decline sharply in number, although remaining significantly higher than the normal; the latter will be readily seen when the standard errors of the mean (σm) are analyzed in table 1.

TABLE 1

Normal rats.

AGE IN DAYS	NO. OF CASES	TYPE I	TYPE II	NON-VESICULAR MEAN AND σM		TYPE III	σM	VESICULAR % OF TOTAL
15	7	334	613	947	7.1	51	0.96	5.1
21	7	224	554	778	12.5	111	0.76	12.5
25	7	226	486	712	22.1	48	1.10	6.3
28	6	183	424	607	20.0	108	1.10	15.0
31	6	165	342	507	6.9	85	1.50	14.4
36	6	120	196	316	4.5	118	0.89	27.1
				Vitamin A-Series				
15	5	306	635	941	30.1	43	1.50	4.4
21	7	354	582	936	25.8	23	1.20	2.4
25	6	254	536	790	18.1	27	0.83	3.3
28	6	215	504	719	10.0	30	1.30	4.0
31	6	242	345	587	10.2	37	1.45	5.9
36	7	218	200	418	4.1	42	1.00	9.1
				Viamin E-Series				
15	5	327	598	925	10.4	58	1.25	5.9
21	6	219	693	912	9.4	170	1.00	15.7
25	6	99	241	340	10.1	94	1.35	21.6
28	6	73	259	332	5.4	116	1.40	25.9
31	6	80	277	357	5.9	131	1.30	27.0

It is apparent that the number of type II follicles exhibits relatively small increases over the normal, except on the twenty-fifth and twenty-eighth day. Type I follicles, however, show consistently larger differences, especially on the twenty-first, thirty-first and thirty-sixth days. It is of interest that the smallest increases in these follicles occur on the twenty-fifth and twenty-eighth days, at which time the most significant increases in type II follicles were observed; that these may be compensatory reactions is suggested by the data, and will receive further consideration in our discussion. The increases in the means of type I and type II follicles thus produce a significant change in the total number of non-vesicular follicles (fig. 1). The major factor responsible for this, however, is the relatively high number of type II follicles in all of the age groups studied.

The means determined for type III follicles during this experimental period (table 1), show that these fall to the twenty-first day, and rise gradually toward the thirty-sixth day. This consistently low level is in sharp contrast to the fluctuation of means in the normal series (fig. 1). The decline in the latter on the twenty-fifth day results in the only small degree of difference between the normal and the experimental animals, with respect to the number of type III follicles. The approximation of all of the means on the fifteenth day may be explained by the observation made on the preceding page.

The above changes in the number of non-vesicular follicles result in a considerable modification of the ratio between these and the type III follicles. This is strikingly evident when the percentage composition of the total number of follicles is computed (fig. 2). It will be noted that the percentage line of type III follicles is similar to that depicting the changes in the total count of this group of follicles. The significantly high percentage line of the non-vesicular follicles is the item of greatest interest, however, in this graph. Comparison between the normal and the experimental series will illustrate the important points of difference. In figure 1, although the non-vesicular means of rats receiving vitamin overdoses is higher than the normal, the same relative decrease occurs throughout most of the stages studied; between the twenty-fifth and thirty-sixth days, the lines are almost parallel. In figure 2, the lower percentage of non-vesicular follicles in normal animals is depicted by the fluctuating line, as contrasted to the relatively uniform and markedly higher line of the experimental rats. In the latter, these follicles form over 90% of the total number of follicles in any age group; the change between any two consecutive days, furthermore, is of a small order of magnitude. From table 1, it may be observed that the percentage differences vary from a maximum of 3.9%, between the thirty-first and thirty-sixth day, to a minimum of 0.7% between the twenty-fifth and twenty-eighth day. The facts acquire additional significance in the light of the low standard errors obtained for all of these groups.

The results obtained by the administration of overdoses of vitamin A to rats stimulated by light, may thus be summarized: (a) the number of type I and II follicles is raised significantly, and the number of type III follicles maintains a uniformly low level; (b) the type I follicles constitute a consistently large proportion of the total number of follicles; (c) the high level of non-vesicular follicles in the experimental animals does not produce a correspondingly significant increase in the total number of follicles present in the ovary at any given time.

Light and vitamin E

The means determined for the total follicle count in animals receiving an oversupply of vitamin E show a 9.2% increase between the fifteenth and twenty-first day, followed by a sharp decline to the twenty-fifth day; in subsequent age groups, the increased number of follicles represents only a 1.03 and a 1.09% increase. Thus, following the high level on the twenty-first day, the total number of follicles in this experimental group is lower than the normal.

In plotting the means of the non-vesicular follicles (fig. 1), the sharp decline between the twenty-first and twenty-fifth day of postnatal life is similar to the trend of the total count; the abrupt change between this age group and that on the thirty-first day, is also repeated in this graph. However, the non-vesicular mean exhibits a 1.02% decrease on the twenty-eighth day, followed by a 1.07% increase on the thirty-first day; a true parallel between the non-vesicular and total follicle count, therefore does not exist.

It is to be expected that the total number of non-vesicular follicles will have considerable influence on the trend exhibited by the relatively low number of all types of follicles present in this experimental series. It is evident, however, that the significant changes in the means of vesicular follicles has a marked effect on this relationship (fig. 1). This is first evident on the twenty-first day, when the non-vesicular means is lower (912) than that of the vitamin A series (941 follicles), although the total count is considerably higher than that established for the latter group of animals; this interesting change is apparently due to the presence of a large number of type III follicles at this stage.

The fluctuating and consistently high number of type III follicles, as contrasted to the normal, is significant at all stages with exception of the twenty-eight day; on this day, it will be noted that the relatively small difference between the means of the experimental and that of the normal series is due to the sharp rise in the latter, rather than to a correspondingly marked decline in the former. The statistical data on table 1 confirm the fact that the effects of the increased number of vesicular types on the total count are real and significant factors in this experiment.

The graph depicting the changes in percentages which each group of follicles constitutes of the total number present in the ovary at various stages (fig. 2), clearly illustrates the fundamental features of the effect of overdoses of vitamin E on the follicular mechanism of the rat. The almost uniform and sharp rise in the percentages of type III follicles is in marked contrast to the fluctuating line of the normal series. Between the fifteenth and twenty-eighth days the increase in percentage composition of the total count is observed to form a straight line; this significant ratio is also reflected in the corresponding decrease in number of the non-vesicular types.

The graphic representation of changes in percentage values would be of less significance without the supporting evidence obtained from our study of the changes in the total number of follicles in the ovary at corresponding age levels; with this additional data it is apparent that the vesicular follicles produce a real and profound change in the total follicle count, and affect the relative importance of variations in non-vesicular follicles in these modifications of the follicular apparatus. If the number of type III follicles remained at a level comparable to that of the normal series, furthermore, the percentage lines of the experimental series would not show such a significant variation from the normal; in this event, both the non-vesicular count and the total follicle count would exhibit a trend which would be comparable to that obtained from our observations on the variations in corresponding means of the normal series.

The outstanding features apparent in this study of the effect of vitamin E on follicular development in the rat ovary, may be summarized as follows: (a) there is a marked and uniform increase in the number and percentage values of the vesicular type of follicles, and this constitutes the most significant and constant factor in this series; (b) the total number of all types of follicles present in the age groups reported is lower than that of the normal rats, and is accompanied by a correspondingly low level of non-vesicular types; (c) the most important factor in producing the latter is the small number of type I follicles in any given age group.

In bringing together some of the data for both the normal and the experimental series of animals, it becomes apparent that the means of the non-vesicular follicles in each case seem to parallel the changes in the total follicle count at corresponding stages; this would be expected because of the large number of these types of follicles present during the early stages of development. In certain cases, however, as noted in the vitamin E-series, this parallel is not striking when the relative numbers of the various follicle types are analyzed. Furthermore, the difference in follicular count between two consecutive age groups of the vitamin A-treated rats and of either the normal or vitamin E groups at corresponding stages, ranges from a minimum increase of 0.3% to a maximum increase of 47%. It is also clear that the insignificant change between any of these means on the fifteenth day of postnatal life, is due to the short time permitted for the action of the vitamin. It is probable that similar data on rats subjected to light in any experimental study of this nature, would also exhibit this insignificant difference. The eyes of the rat open between the fourteenth and fifteenth day, and consequently the time available for light stimulation would be insufficient to produce an appreciable change in the follicle count.

The fluctuating line obtained from the means of vesicular follicles in the vitamin E-series, is of importance because of its similarity to that depicting corresponding changes in the normal rats. The characteristic features of the former series, however, are noted in comparison with rats receiving overdoses of vitamin A; the minimum number of vesicular follicles in the vitamin E group is never as low as that recorded in either the normal or in the vitamin A group. The minimum difference is 0.7% and the maximum difference is 86.5%.

Finally, it is of interest to note in comparison of table 1 and figure 2 that the data obtained from the normal animals occupy an almost exact middle position between the vitamin A and the vitamin E series. The non-vesicular percent of the normal rats ranges from 3% to 3.2% below and above, respectively, that of animals treated with vitamins A and E; furthermore, the same range of difference above and below the experimental series is observed in the graph depicting changes in the vesicular percentage of rats under normal laboratory conditions (fig. 2). These variations of follicle types around the normal means is of utmost import in this study, since it represents the significant effects produced by these vitamins on the follicular picture of the normal rat.

DISCUSSION

The specific action of vitamins A and E on the follicular mechanism of the rat clarifies the concepts formulated from the more general studies on the relation of vitamins to sexual reproduction. The data reported above is of further import in the bearing it has on our continued analysis of the physiological factors operating in hypophysial-gonadal interaction.

It is clear that these vitamins produce a modification distinct from that induced by light, and that the augmentation effect on the different types of follicles is characteristic of the individual vitamin. Overdosage of vitamin A results in an increased number of non-vesicular follicles (types I and II), without altering significantly the total number; the immediate result of this increase is that these follicles constitute a uniformly higher percentage of the total than that observed in any of the other series. The consistently large number of type I follicles, relative to that observed in the normal rat, is of interest. With the increase in non-vesicular types, furthermore, there is a significantly low level of vesicular follicles (type III).

Overdosage of vitamin E operates by increasing the number of type III follicles, which constitute a higher percentage of the total than do the vesicular follicles of the other series. The non-vesicular types, however, drop sharply in number between the twenty-first and twenty-fifth days; after this, the number remains relatively constant but significantly below the normal. It is obvious that these changes will modify the total count at any given stage; the parallel that would exist between nonvesicular and total count, however, is greatly altered by the constant and significant rise in the number of type III follicles. This is particularly evident on the twenty-first day when although the non-vesicular number is less than that of the vitamin A-treated rats, the total number is considerably greater due to the high level of the vesicular follicles.

In comparing the relative proportion of the different types in the normal series, a noteworthy feature is the alternating ratio between them, with the type III follicles decreasing this ratio markedly in the later stages. As these follicles gain ground during the succeeding weeks, the apparent struggle will reach a climax, and, under the appropriate conditions, the first estrous cycle will ensue.

These phenomena might be related largely to the availability and utilization of substances necessary for growth processes, on the one hand, and for the final stages of growth and maturation, on the other. When the availability of these substances is altered by changes in either the external or internal environment, the effect on growth and maturation would be reflected by deviations of the follicle types from the normal mean. The generalized nature of stimulation by light (Truscott, '44 b) might be expected, therefore, to affect both the earlier and later developmental stages of the follicles. This is supported by our observation that the number of non-vesicular follicles increases over the normal, and that the level of vesicular follicles fails to show wide fluctuations characteristic of animals under normal laboratory conditions.

The results obtained by furnishing an oversupply of vitamin A or of vitamin E, in addition to light stimulation, acquire further significance from the above discussion. The means of the total non-vesicular counts and the percentage values of these in rats placed under continual illumination fall between the normal series below and the vitamin A-series above. Corresponding values of the vesicular follicles in the continual light series fall between the normal and vitamin E-series in the same manner. The specific character of the effects described in the present experiment thus strongly indicates the important role of vitamin A in the growth processes, and of vitamin E in the maturation processes of the ovarian follicles.

The possibility that we are dealing here with an inhibitory effect on the growth of one type of ova, rather than with a stimulatory effect on that of another type, should receive consideration. The total counts presented in figure 1 might be interpreted as a direct inhibition of the fluctuating trend of the vesicular follicles in the normal animal. The significant increase in number of non-vesicular types in the vitamin A-series, however, negates such an interpretation. The greater number of type I and II follicles results in an increased utilization of the available material for growth processes of all types of follicles; this will produce a corresponding depletion of the supply of vitamin A necessary to the increasing metabolic activities of the older follicles.

Further evidence for this belief is adduced when we consider the possibility that vitamin E may inhibit the growth of the younger follicles. If this were the case, the number of vesicular follicles would not differ appreciably from the normal; but the significant and constant rise in number of these follicles operates against this interpretation. The increased number of maturing follicles constitutes an increasingly large demand for substances necessary for completion of the later stages of growth; this will produce a marked effect on the fat of the younger follicles, as seen in the sharp drop in the latter.

It is apparent that we are dealing with a complex process in which vitamin A and vitamin E act in a specific manner on the growth and maturation of ovarian follicles. In interpreting the data of this experiment, one should bear in mind that an oversupply of one vitamin is present in animals which possess an adequate supply of the other vitamin. In the rats treated with overdoses of vitamin E, the larger number of non-vesicular follicles deplete a sufficient amount of vitamin A to operate against a still more pronounced increase in the number of vesicular follicles. In the vitamin A-series, the available supply of vitamin E may not be utilized efficiently by the vesicular follicles since they do not attain the stage where this vitamin may act in its characteristic manner; few follicles reach the transition point between non-vesicular types due to the depletion of vitamin A by the greatly increased number of younger follicles.

The belief that these vitamins may function in ovarian development in the specific way described above, receives direct and indirect support in the literature. It has been demonstrated (Euler and Schmidt, '34) that purines, necessary as building units of nuclei, increases when vitamin A is administered to tissues formerly growing under conditions of vitamin A-deficiency. Further evidence, discussed in particular by Rosenberg ('42), illustrates the important role of this vitamin in cell growth.

Increasing evidence supports the concept that vitamin E is intimately linked with processes of cell maturation (Juhász-Schäffer, '31; Mason, '33: and others). The use of vitamin E in healing of skin wounds, due to the rapid proliferation of the tissue, and in growth of prematurely born animals has been discussed elsewhere (Adamstone, '31; Léranth and Frank, '36; Rosenberg, '42).

The distribution of these vitamins in the body of the normal animal is of considerable import. Popper and Greenberg ('40) and Popper ('41) have shown that vitamin A is present in the tubular and Leydig cells of the testes, in the granulosa and theca lutein cells of the ovary, and in the anterior lobe of the pituitary; this distribution is affected by the physiological state of the gonads. The distribution of vitamin A in the liver and adrenals, on the other hand, is influenced by the age and nutrition of the animal. It has also been demonstrated that vitamin E is present in high concentrations in the placenta and anterior lobe of the pituitary body (Evans and Burr, '27; Léranth and Frank, '36; and others). The presence of these vitamins at these points in the hypophysial-gonadal mechanism, and the modifications in their distribution during the sexual cycle of the animal, indicate a correlation between their availability and subsequent utilization in determining the reactions reported in the present study.

In presenting the second part of our investigation of physiological factors underlying hypophysial-gonadal interaction, we are led to believe that vitamins A and E play a far more important and specific role in this relationship than has been hitherto admitted. It may also be inferred that the general activation by light (Truscott, '44 b) operates by releasing, or in some manner affecting the distribution of these vitamins. This modification in availability of compounds important in follicular growth and maturation, is apparently reflected in the results discussed above. It is still a matter of conjecture, however, as to whether these phenomena involve an opto-hypothalamo-pituitary pathway (Truscott, '44 a). Further morphological and experimental investigation of this is in progress.

The action of the follicle-stimulating hormone and the luteinizing hormone on the rat ovary (Lane, '35) affords a remarkable parallel with the effect of vitamin A and of vitamin E, respectively, as noted in the present study. These facts, and others arising from the above discussion, strongly indicate the necessity for a thorough revision of the hypophysial-gonadal relationship which is based on our present concept of a gonadotropic-hormone mechanism. It is apparent that the solution to many of these problems may be reached by further analysis of vitamin release, availability, and utilization in the body. Experiments are now in progress to determine more precisely the extent to which these vitamins participate in this phenomenon of interaction.

SUMMARY AND CONCLUSIONS

1. Albino rats received oversupplies of vitamin A or of vitamin E at various stages throughout early postnatal life. The animals were then subjected to a controlled amount of visual stimulation.

2. The ovarian follicles were counted in individual members of each group, and typed according to a scheme outlined by the author in an earlier study: Nonvesicular follicles included (a) type I, with one layer of granulosa cells, and (b) type II with two or more layers of granulosa cells and without antra. Vesicular follicles constituted the type III group, having one or more antra.

3. The number of non-vesicular follicles in rats receiving overdoses of vitamin A is significantly higher than the normal, although the number of type III follicles maintains a uniformly low level. The large number of non-vesicular follicles does not produce a corresponding increase in the total number of follicles.

4. The vitamin E-treated rats show a striking and constant increase in the number of vesicular follicles; the number of non-vesicular follicles in general, and of type I in particular, is markedly lower than the normal. These changes produce a significantly lower number of all types of ovarian follicles at any stage of this series.

5. The results of our investigation indicate the important and specific role which these vitamins may play in hypophysial-gonadal interaction.

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THE ACCESSORY TENDON OF THE FLEXOR POLLICIS LONGUS MUSCLE

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TWO FIGURES

The flexor pollicis longus muscle is described as arising chiefly from the ventral surface of the radius from a point slightly below the bicipital tuberosity to one within 5 cm. of the wrist, and from the neighboring portion of the interosseous membrane. It is also stated that the muscle also derives additional origin from the medial border of the coronoid process of the ulna by means of a delicate fleshy bundle, As a variation, the latter origin may be prolonged proximally to the medial epicondyle of the humerus.

The fiber-bundles of the main portion of the muscle descend obliquely to be inserted in a penniform manner on a tendon which begins high on the volar surface near the ulnar border of the muscle. At first broad, it becomes cylindrical near the wrist. The insertion of fibers continues nearly to the point where the tendon passes under the transverse carpal ligament.

In the current report the authors are concerned with the frequency of occurrence of the accessory tendon and with its form and attachment.

MATERIAL AND METHODS

For this study 150 consecutive upper extremities were examined, most of them in pairs from cadavers of adult American whites and negroes (chiefly male). Three specimens were selected for illustrations (figs. 1 and 2, of right forearms); in 21 the accessory tendon was followed to its skeletal origin.

OBSERVATIONS AND DISCUSSION

In all specimens studied, the radial and interosseous (membranous) heads of the muscle were present, the fascicles from these portions descending obliquely to converge upon the tendon in penniform fashion (fig. 2b, at 1 and 2). In 80 of the 150 specimens, an accessory head

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was present, this narrow band appearing as a prolongation toward the medial condylar area of the brachium (fig. 2b, at 3). It was brought into view upon reflection of the flexor digitorum sublimis muscle. Commonly widening as it ascended, the tendon became part of the muscle mass which employs the humeral condyle (fig. 1b), or the ulnar coronoid process (fig. 2a), for origin. In every instance the accessory tendon was intimately fused with other muscles of the flexor-pronator group (figs. 1a and 1b).

In specimen I the accessory tendon was fused laterally with the deep portion of the pronator teres (fig. 1b), medially with the flexor digitorum sublimis and flexor carpi ulnaris — to the former through unipenniform fascicles. On intermediate level, then, three muscle-heads took origin from a narrow line (5 mm. wide) on the summit of the medial epicondyle of the humerus: flexor pollicis longus; humeral head of the flexor digitorum sublimis; humeral head of the flexor carpi ulnaris. On superficial level these are bound in with the superficial head of the pronator teres, the flexor carpi radialis and the main mass of the humeral portion of the flexor digitorum sublimis and the palmaris longus (fig. 1a). These elements were so intimately fused that segregation could be accomplished only by cutting along their tendinous portions proximalward from the point at which they radiated to separate insertions.

In specimen II the accessory tendon of the flexor pollicis longus arose from both the humerus (cf. specimen I) and the ulna (cf. specimen III), the origin being held in common with that of the deep head of the pronator teres, in an elongate stripe at the medial margin of the coronoid process. As the tendon crossed the joint, from epicondyle to coronoid process, it blended with the fibrous tissue of the articular capsule.

In specimen III the accessory tendon was likewise fused with the deep head of the pronator teres (figs. 2a, 2b). However, instead of arising from the humerus, it took origin from the coronoid process of the ulna just medial to the area of insertion of the brachialis. Descending obliquely radialward, the tendon was joined first by fascicles of the radial head, then by those of the interosseous portion (figs. 2b and 2c, respectively at 3, 2 and 1). The tendon narrowed after leaving the

Fig. 1 Specimens I. a, accessory head of the flexor pollicis longus in relation to other muscles (cut) of medial epicondylar origin. The accessory tendon here emerges from beneath the flexor digitorum sublimis (lifted by string). The accessory tendon is of humeral origin. 1b, deep relations of the accessory head, shown by cutting the brachioradialis and extensores carpi; the biceps brachii and brachialis: the flexor carpi radialis, superficial head of the pronator teres, palmaris longus and flexor digitorum sublimis.

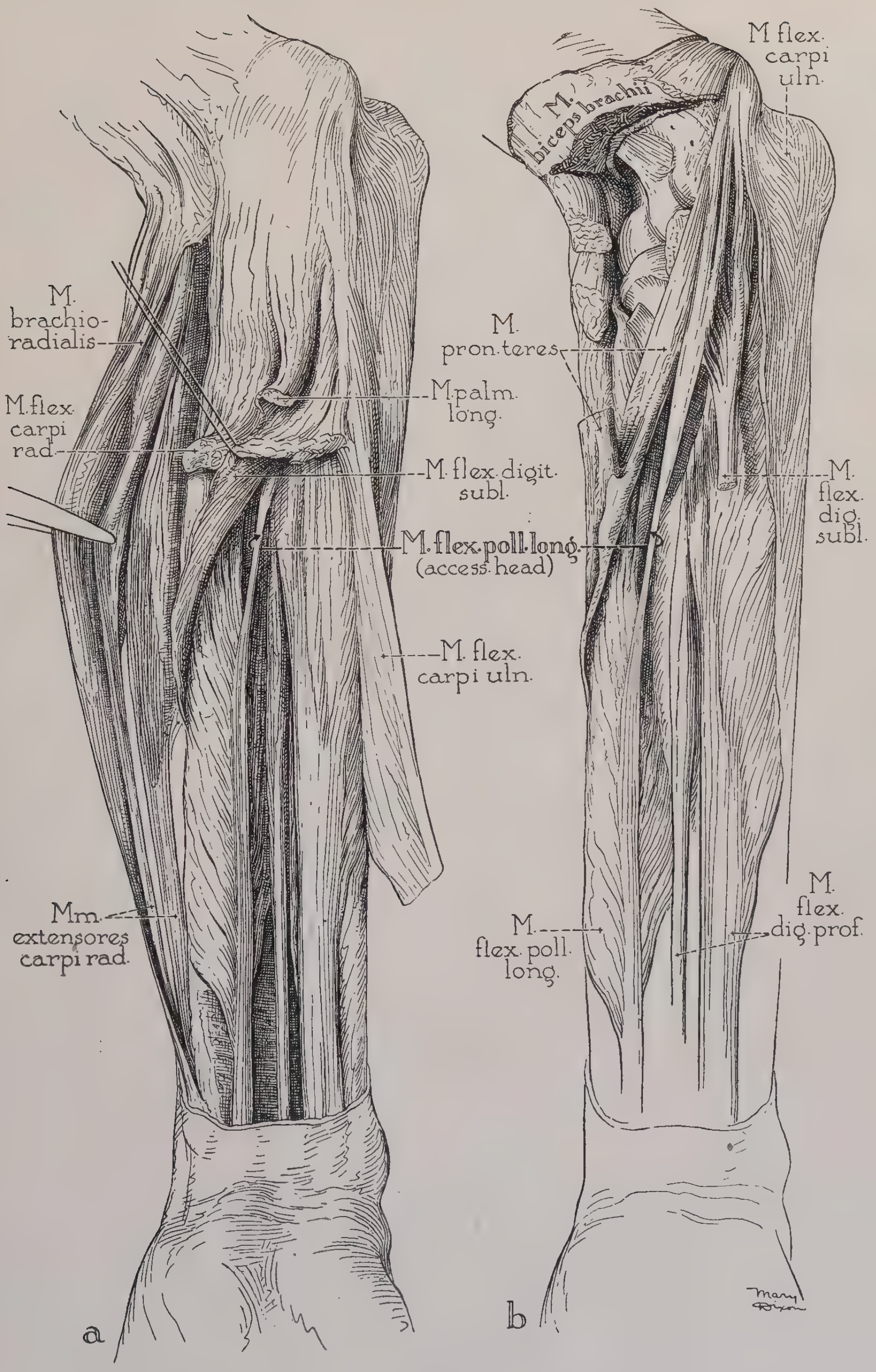
M. brachio-radialis
M. flex. carpi rad.
M. palm. long.
M. flex. digit. subl.
M. flex. poll. long. (access. head)
M. flex. carpi uln.
Mm. extensores carpi rad.
a
M. biceps brachii
M flex. carpi uln.
M. pron. teres
M. flex. dig. subl.
M. flex. poll. long.
M. flex. dig. prof.
b
Mary Dixon

Figure 1

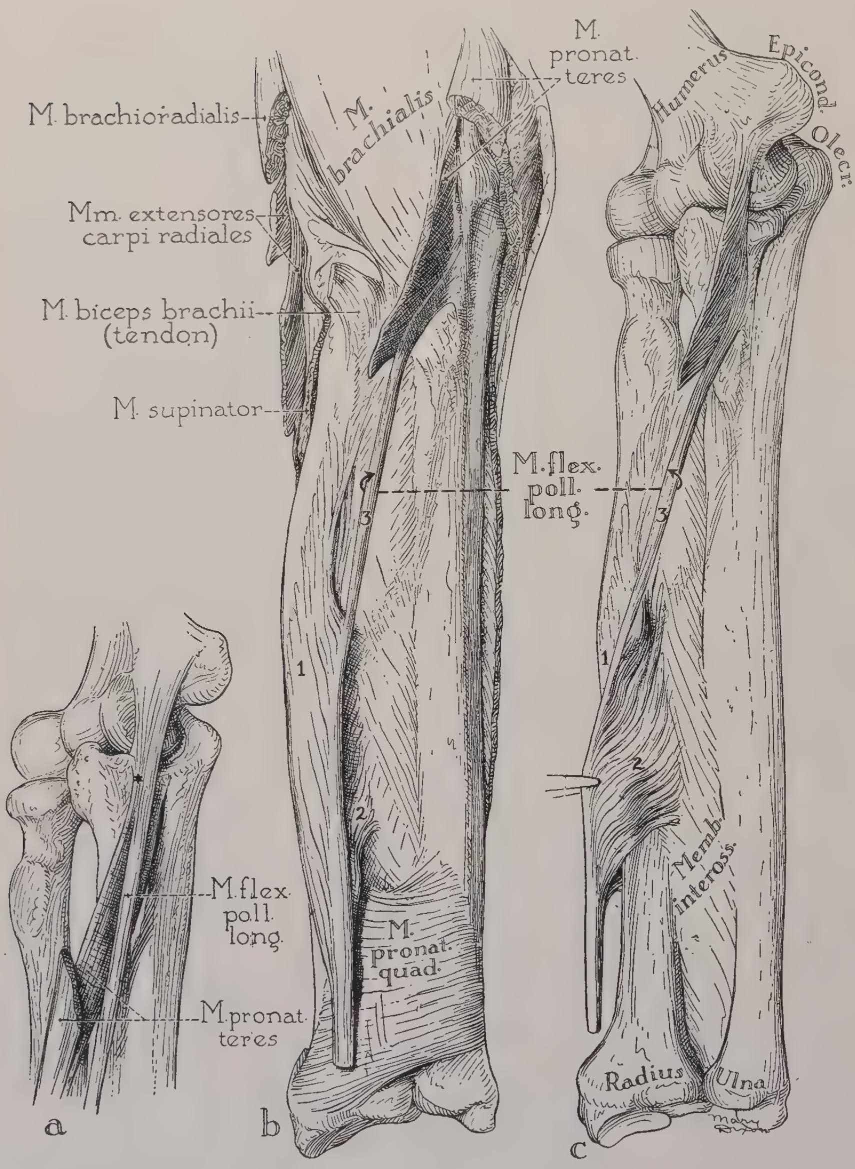
M. brachioradialis
M. brachialis
M. pronat. teres
Humerus
Epicond.
Olecr.
Mm. extensores carpi radiales
M. biceps brachii (tendon)
M. supinator
M. flex. poll. long.
3
3
1
1
2
2
Memb. inteross.
M. flex. poll. long.
M. pronat. quad.
M. pronat. teres
Radius
Ulna
a
b
c

Fig. 2 Specimens II and III. a, specimen II, deep dissection, to illustrate humeral (epicondyle) and ulnar (coronoid process) origins. b, specimen II, deep dissection, showing the flexor pollicis longus with radial (at 1), membranous (2) and ulnar, or accessory (3), heads. The muscles of humeral origin have been cut, the flexor digitorum profundus removed entirely. c, specimen II, continued, showing the course, and muscular contributions to, the tendon of the flexor pollicis longus (retracted). The pronator quadratus has been removed, as have all muscles of humeral origin except the deep head of the pronator teres.

ulnar head of origin, to become band-like where it ceased to receive muscle fibers; next, it gradually widened and thickened as it received fibers from the bone and the interosseous membrane. The radial head was twice as long as the interosseous. The former descended through a greater distance before becoming implanted into the muscle (fig. 2a) than did the latter (fig. 2b). The interosseous head extended half-way across the membrane, ulnarward. Like the radial head it was unipenniform. It used for origin the middle third (vertically) of the interosseous membrane, while the longer radial head employed the middle two-fourths of the radius. Thus, the radial head was the strongest portion of the muscle, while the ulnar portion was weakest — the last-named having but few fasicles independent of those of the adjacent pronator teres muscle.

Of the total lot of 150 extremities (77 right, 73 left), 80 possessed the accessory tendon (42 right, 38 left); 54 of the 80 were instances of bilateral occurrence (in 27 cadavers).

By tracing the accessory tendon to its proximal attachment in 21 specimens (including those illustrated), it was found to have a humeral origin in all but one (specimen III), where its origin was solely ulnar, both humeral and ulnar origins in 11 (as in specimen II). In every case it took origin in part from the articular capsule in a narrow strip between the medial epicondyle of the humerus and the coronoid process of the ulna. Minor attachments occurred occasionally, as follows: to the tendon of insertion of the brachialis (in 6 cases), to the oblique cord of the radius (1 case) and the deep head of the pronator teres (3 cases).

CONCLUSIONS

The so-called accessory tendon of the flexor pollicis longus is present more frequently than it is absent; an origin from the humerus is almost always present, an additional one from the ulna occurs in about one-half of the cases. These conclusions are based upon a study of 150 consecutive extremities, in which it was found that 80 possessed an accessory tendon of origin (about equally distributed between right and left sides, and in 27 cadavers bilaterally present). The regular skeletal origin of the elongate tendon was found to be the medial epicondyle of the humerus; the medial side of the coronoid process of the ulna offered secondary bony origin in 11 of 21 completely dissected extremities, the coronoid alone in one specimen. Invariably the tendon also employed the capsule of the elbow joint for attachment. Occasionally lesser slips passed to the tendon from neighboring tendinous structures.

BOOK REVIEW

PROCEEDINGS OF THE CONFERENCE ON PROBLEMS OF HUMAN FERTILITY. Sponsored by the National Committee, on Maternal Health. Edited by EARL T. ENGLE, General Chairman of the Conference. 1943. v + 182 pages, 25 half-tones. Cloth, $2.00. Paper $1.50.

A grant from the Johnson Research Foundation made it possible to hold the Fourth Annual Conference, January 15–16, 1943, and to publish the Proceedings. The book may be ordered from the Office of the Committee, 2 East 103 Street, New York City 29, but it will be mailed only upon receipt of check (postpaid). It is composed of 11 papers along with 75 pages of discussion by 34 of the 54 individuals who attended. Five of the papers are accompanied by short bibliographies.

The conference was divided into three sessions: Criteria of Ovulation, Philip E. Smith, presiding; Some Biological Observations on the Lower Generative Tract, Elmer L. Sevringhaus, presiding; and The Placenta as a Barrier, George W. Corner, presiding.

The following are the eleven papers which were presented: Endocrine Criteria of Ovulation, Elmer L. Sevringhaus; Embryological Criteria as Evidence of Ovulation Time, John Rock and Arthur T. Hertig; The Age of Corpora Lutea and Timing of Ovulation, William W. Greulich; Electrometric Techniques Correlated with Other Studies of Ovulation, John L. Boling; Some Observations on Sperm Migration Through Cervical Mucus, Jule K. Lamar; The Transportation and Survival of Sperm in the Vaginal and Cervical Canals, Boris B. Rubinstein; Present Problems Related to the Biology of the Vagina, A. E. Rakoff; Some Observations on the Cytology of the Trophoblast, George B. Wislocki; Placental Transmission in Relation to the Stage of Pregnancy, Franklin P. Snyder; Placental Transmisson of Radioactive Isotopes, Alfred Gelhorn; and The Passage of Native Proteins Through the Placenta, Bret Ratner.

Regarding ovulation in women, it was said at conference that no technique which is now available may be expected to reveal the precise moment when it occurs. Criticised from the floor were the original interpretations of the best case yet to be studied by means of the electrometric technique (Yale J. Biol. and Med., 1937, vol. 10, p. 155). It was argued that the change in electric potential which had been attributed to ovulation must have occurred several days after the follicle had ruptured.

Using the histology of the corpus luteum as a basis of final judgment, evidence was presented that ovulation is associated with changes in the temperature of the body which occurs near the middle of the menstrual cycle. The follicle ruptures after the temperature has begun to rise from a previous low. Mid-menstrual pain (Mittelschmerz) may be due to such phenomena as contractions of the Fallopian tubes in response to estrogen, and not to the actual process of ovulation. New evidence for this is the fact that in the one patient who was observed the pain ceased before the temperature became elevated.

On the basis of embryological criteria (estimating age of ovum), it was reported that ovulation occurs about 14 days before the "next expected menstruation." The finding that the post-ovulatory portion of the cycle is relatively uniform in length is contrary to earlier observations in the rhesus monkey.

There was ample refutaton of the contention that cornified cells in the vaginal smear during menstruation reflect the occurrence of ovuation while bleeding is in progress. Such cells become exfoliated from the intermediate layer of the vaginal epithelium in response to diminished quantities of ovarian hormones. An erratum appears on page 88 and again on page 89 where this layer is designated the "intermediate zone of Dierck." It would seem that reference was being made to the "intraepitheliale Verhornungszone" of Dierks (Arch. f. Gynäk., 1927, vol. 130, p. 46 and 1929, vol. 138, p. 111).

Although there was general agreement that the determination of pregnandiol cannot as yet be used as a technique for estimating the time of ovulation, the question was raised as to whether the "excretion of pregnandiol during non-ovulatory cycles" could be due to the formation of progesterone by ovarian follicles. Whereas the evidence accumulates that previous to rupture the normal follicle may exhibit early stages of luteinization, observations in the opossum suggest that atretic follicles are not sources of progesterone.

Spermatozoa, in vitro, were able to penetrate cervical mucus which had been collected on days 8 to 14 of the menstrual cycle but they failed to penetrate mucus that was secured on days 16 to 26. In women who were presumably sterile, it was found that after coitus the spermatozoa within the vagina did not survive longer than 3 hours.

Concerning the human placenta, it was postulated that the syncytial trophoblast is "probably the site of synthesis and secretion of chorionic progesterone and estrogen." This is compatible with the absence of these hormones in cultures of placental tissue which contain chorionic cells but not syncytium (Bull. Johns Hopkins Hosp., 1943, vol. 72, p. 26). In considering the brush-border of the syncytiotrophoblast, two opposing views are expressed: (1) that it reflects the taking up of fluid from the intervillous space ("pinocytosis") and (2) that it owes its origin to the rupture of peripheral vacuoles which had been produced by the liquefaction of secretory granules. Recently, both views have received fuller consideration (Am. J. Anat., 1943, vol. 73, p. 335 and 1944, vol. 74, p. 291). Although immunological studies indicate that certain proteins traverse the placenta, notably the Rh factor, it is still uncertain whether they pass through the intercellular spaces of the placental barrier or only through such gaps in the barrier as those which are formed when chorionic villi become detached. The inadequacy of the term "placental barrier" was expressed admirably by the presiding chairman when he said, "It is perhaps not too fantastic to compare the placenta to a subway turn-stile, which lets you through, but only if you are not too wide and if you have the right fare."

In certain non-primates, the placenta was found impermeable to such hormones as epinephrin, insulin, parathormone, pituitrin, an extract of the anterior hypophysis and an extract of pregnancy urine. The rate of transfer of radioactive sodium across the placenta was approximately 320 times faster in the hemochorialis type of placenta than in the epitheliochorialis type. There was failure to confirm previous reports that during late stages of pregnancy the blood in the umbilical vein of the fetus is little more than half saturated with oxygen. Near term, when the tendency of the uterus to contract was inhibited by injections of hormones, the saturation of oxygen in the blood of both fetus and mother was found to be about 90%.

Returning to the book as a whole, it is an important contribution to the physiology of human reproduction. Its outstanding features are the appraisal of research in this field and the resultant light shed on problems for future study. These features emerged during informal discussion and they illustrate the benefits which may arise from a gathering of seasoned investigators.

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OCCURRENCE OF FOLLICULAR CYSTS IN THYROIDECTOMIZED RATS TREATED WITH DIETHYLSTILBESTROL

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ONE PLATE (FIVE FIGURES)

The formation of follicular cysts in ovaries may be brought about by any one of several experimental procedures and is apparently caused by an imbalance of the gonadotrophic hormones. The most common method which has been used is the administration of gonadotrophic preparations, particularly the follicle stimulating factor. It is likely that the increase in the incidence of follicular cysts induced by testis implants (Pfeiffer, '36), the injection of testosterone in young rats (Shay et al., '39) (Wilson, Hamilton and Young, '41), the combined treatment with estradiol and progesterone in baboons (Gillman, '42), or in parabiotic rats when one of the mates has been castrated (Hill, '32), results from modification in the levels of gonadotrophic hormone rather than by a direct action of the various substances or procedures on the ovary. Recently, Starkey and Leathem ('43) caused the formation of cystic ovaries in kittens by the administration of pregnant mare serum.

Ovaries of thyroidectomized animals show some variation from ovaries of normal animals. Hammett ('26) and Zeckwer ('38) found that the ovaries of rats which had been thyroidectomized for several weeks were smaller than those from normal animals. Leonard and Leonard ('37) found no significant change in the ovaries of immature rats which had been thyroidectomized for 6 to 8 days. On the other hand, Chu ('44), pointed out that in thyroidectomized rabbits the ovarian follicles were more numerous and larger than in control animals.

The present observations on cystic ovaries were made on rats which were being used for studies on the metabolism of carbohydrates.

METHODS

Rats of the Long-Evans strain, which had been inbred in our laboratory for many years, were used for these experiments. Three groups of animals were studied.

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1. Rats that had been thyroidectomized for 8 months were given daily subcutaneous injections of 100 micrograms of diethylstilbestrol for the last 20 days.
2. Rats that had been thyroidectomizd for 8 months were given subcutaneous implants of two 10-mg. pellets of diethylstilbestrol (95% diethylstilbestrol and 5% methyl cellulose). The first pellet was implanted at the time of thyroidectomy and the other 6 months later.
3. Rats that had been thyroidectomized for 1 month were given daily injections of 100 micrograms of diethylstilbestrol for the last 20 days.

Control animals were studied in each series.

The animals were fasted for 36 hours before they were killed and the ovaries were fixed in either Flemming's solution or Zenker's-formol solution. The pituitaries were fixed in Zenker's-formol solution and stained with the Mallory-azan technique. A check was made for thyroid remnants in the operated animals and a small amount of regenerated tissue was found in three animals. However, these animals were included in the study since they showed no differences from those in which the operation was complete.

OBSERVATIONS

Rats in our colony rarely exhibit cystic ovaries following estrogen therapy in any amount or for any duration. One of eight animals, however, had cystic ovaries after it had been thyroidectomized for 8 months and one of five rats had follicular cysts when it had been thyroidectomizd for 8 months and given constant estrogen stimulation with implanted stilbestrol pellets. The incidence of cystic ovaries was further increased when seven of ten rats, which had been thyroidectomized for 8 months and given stilbestrol the last 20 days, showed follicular cysts (see table 1).

TABLE 1

Occurrence of cystic ovaries in rats.

(Long-Evans strain)

TREATMENT	NUMBER OF RATS	NUMBER OF RATS WITH CYSTS
Normal	18	0
Normal, stilbestrol for 20 days	8	0
Normal, stilbestrol pellets for 8 mo.	5	0
Thyroidectomy for 8 mo.	8	1
Thyroidectomy for 8 mo., stilbestrol pellets for 8 mo.	5	1
Thyroidectomy for 1 mo.	6	0
Thyroidectomy for 1 mo., stilbestrol last 20 days	8	0
Thyroidectomy for 8 mo., stilbestrol last 20 days	10	7

When the cystic condition occurred there were from one to four follicular cysts in a given ovary. The cysts were transparent structures easily visible to the naked eye. Microscopically they appeared as sacs filled with follicular coagulum (figs. 3 and 4). As a rule the thick granulosa cell lining of the follicle had undergone atrophy and was represented by a single layer of cells, cuboidal to squamous, which rested on a basement membrane. Luteinization of the theca interna occurred adjacent to some of the cysts and encroached upon them (figs. 4 and 5), but the basement membrane and granulosa cells lining the cysts remained intact. In almost every instance ovaries which contained cysts also contained some large corpora lutea and a few small follicles.

The ovaries of rats which had been thyroidectomized for 30 days appeared normal in structure (similar to fig. 1), but when these rats or normal animals were given injections of stilbestrol for 20 days, the ovaries contained, in most cases, large corpora lutea and small follicles as has been noted by Merckel and Nelson ('40), in normal animals. The ovaries of animals which had been thyroidectomized for 8 months and which had received no treatment were smaller, in general, than those of normal animals and had a smaller number of corpora lutea and follicles, the latter being in various stages of development. However, animals, either thyroidectomized or normal, which had received stilbestrol pellets for 8 months usually had atrophic ovaries that contained a few small follicles and many interstitial cells (fig. 2).

The pituitaries of all untreated thyroidectomized rats were increased in size and showed typical large vacuolated basophilic "thyroidectomy" cells. In animals which were thyroidectomized for 8 months these cells were more numerous, larger and showed a somewhat greater degree of vacuolation than did those which were thyroidectomized for only 1 month. The eosinophils were small and showed degranulation.

Following injections of stilbestrol the weights of the pituitaries were further augmented. When these glands were examined microscopically they showed a decrease in the number and size of the "thyroidectomy" cells and in some instances a complete absence of these cells.

In the rats which had implants of stilbestrol at the time of thyroidectomy the pituitaries were very large, "thyroidectomy" cells were absent, the chromophil cells were degranulated and there was an increase in the number of chromophobes.

DISCUSSION

It is generally agreed that the anterior pituitary controls the growth of the graafian follicle, after it has reached a certain stage in its development, controls ovulation and subsequent formation of the corpus luteum. FSH produces follicular development, a combination of FSH and LH causes the rupture of the follicle and LH is required for corpus luteum formation. One would thus suspect that in the formation of cystic follicles there is either an over-production of FSH, or an under-production of LH since the follicles continue to grow and are not luteinized at the appropriate time. The fact that the incidence of cystic follicles is greatly enhanced in thyroidectomized rats treated with stilbestrol would lead one to believe that the estrogen was causing the release of FSH. This point of view is contrary to the accepted opinion that estrogens cause the release of LH from the pituitary (Fevold, Hisaw, Greep, '36).

Of course the pituitaries of thyroidectomized animals are somewhat different, both morphologically and physiologically, from the pituitaries of normal animals and it is entirely possible that they would not react to estrogens in the same manner as do the hypophyses of intact animals. Morphologically, the pituitaries of thyroidectomized animals shows a degranulation of the eosinophils and an increase in number and vacuolation of the basophils. When the pituitaries of thyroidectomized animals are considered from a physiological point of view there is some variance in the observations of different investigators. Stein and Lisle ('42) found a decrease in the gonad-stimulating potency of the pituitaries of young male rats which had been thyroidectomized. Smith and Engle ('30) and Van Horn ('33) found no difference between the gonadotrophic activity of pituitaries from thyroidectomized female rats and pituitaries of normal animals, when implanted into immature mice. However, Chu ('44) made fresh saline extracts of hypophyses from thyroidectomized rabbits and found the extracts to contain more FSH than extracts of pituitaries from normal animals.

It is possible to obtain further evidence concerning the gonadotrophic activity of the hypophysis from work done on the pituitary of castrated animals since the basophilic cells in pituitaries of castrated and thyroidectomized rats are thought to be similar (Nelson and Hickman, '37; Severinghaus, '37), although some recent work (Reese et al., '43) has indicated that there is a slight morphological difference between the vacuolated basophilic cells in the two conditions. It is interesting to note that the pituitaries of castrates contain more gonadtrophic hormone (Engle, '29). Leonard ('37) found that the pituitaries of spayed rats contained increased amounts of FSH, but there was little change in the content of LH.

When estrogens were given to thyroidectomized rats the "thyroidectomy" cells were reduced in number and size and in some cases disappeared completely. If the basophils produce FSH, as has been suggested by Severinghaus, they may also be storing large amounts of this substance following thyroidectomy. If this is true, the action of estrogens in correcting these basophils might cause a release of FSH in relatively large amounts which in turn could cause the production of cystic ovaries.

The ovaries which contained cysts also had large corpora lutea. In fact, the theca interna in some cysts was luteinized. This condition indicates that LH was also being released by the pituitary. Consequently, estrogen may have been causing the release of both FSH and LH, but the FSH probably was given off in such large quantities that certain of the follicles became cystic.

SUMMARY

Cystic ovaries have been observed in rats which have been thyroidectomized for 8 months. The incidence of their occurrence is increased when diethylstilbestrol is given during the last 20 days . However, the ovaries of thyroidectomized animals which have received prolonged treatment with diethylstilbestrol, become atrophic.

The cysts are thin-walled vesicles, usually lined with a single layer of granulosa cells and the theca surrounding some of the cysts is luteinized.

Although estrogens cause the secretion of LH in normal animals, it seems likely that they may cause the release of stored FSH from the hypophyses of thyroidectomized rats, thus accounting for the production of cystic follicles.

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PLATE 1

EXPLANATION OF FIGURES

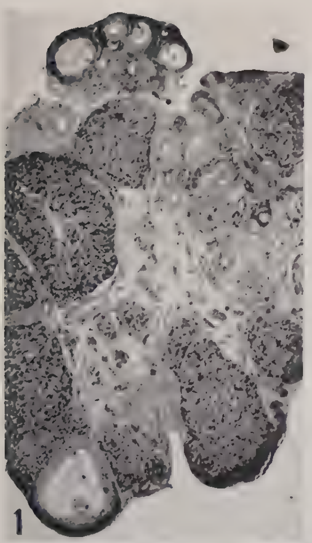
1 Ovary from normal rat containing medium sized corpora lutea and small follicles. × 18.

2 Ovary from rat which had been thyroidectomized for 8 months and had received two implants of diethylstilbestrol pellets during that time. Notice general atrophic condition, absence of corpora lutea and presence of small follicles. × 18.

3 Ovary from rat that had been thyroidectomized for 8 months and received injections of diethylstilbestrol the last 20 days. One clear follicular cyst is present in addition to large corpora lutea and small follicles. × 18.

4 Ovary from rat receiving same treatment as the one in figure 3, but the ovary shows multiple cysts. The cyst on the right is almost completely surrounded with lutein cells. × 18.

5 Enlargement of area blocked off in figure 4, but from an adjacent section. Notice the flattened epithelium lining the cysts and theca luteinization of the cyst on the right. × 88.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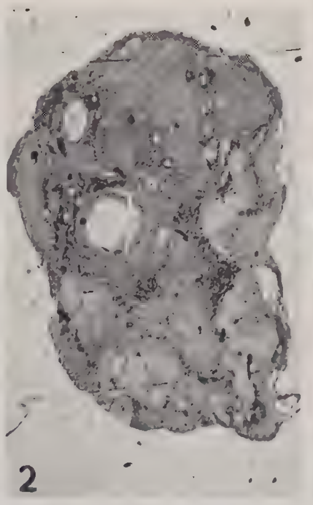
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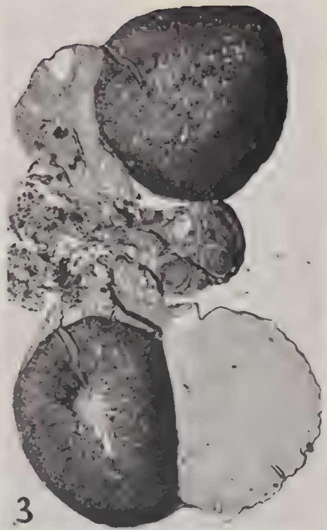
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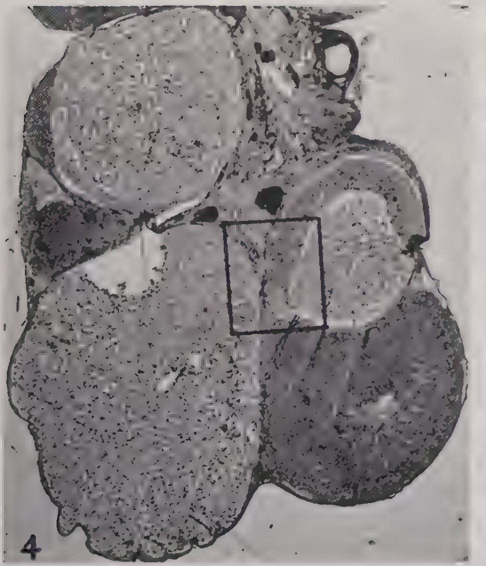
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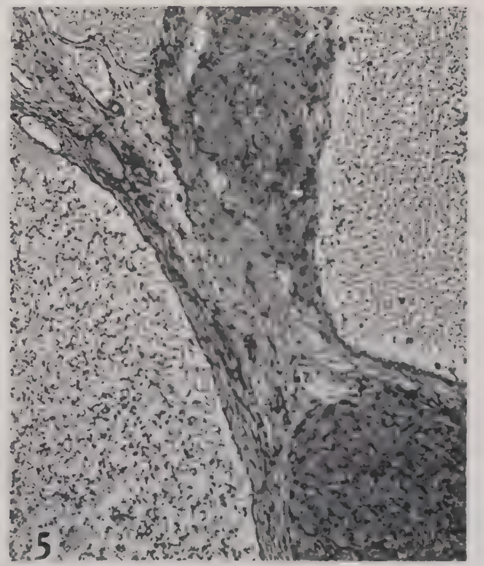
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ON ABNORMAL BLASTULAS IN ELEPHANTULUS

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FIVE FIGURES

In a previous study we have drawn attention to the fact that at ovulation as many as 120 eggs may be liberated from the ovaries in Elephantulus myurus jamesoni (van der Horst and Gillman, '41). In a sterile ovulation, which in this animal is not an unusual phenomenon, all these eggs can be found some time after ovulation in the upper part of the uterine horns, where they are prevented from descending further down by a strong contraction of the uterine musculature, and here they soon disintegrate. When coitus has taken place the majority of the sixty odd eggs in each Fallopian tube may be fertilized and they may start to segment normally. However, only one egg in each horn of the uterus implants itself and continues to develop into a normal embryo. All the others, no matter how normal and healthy they appear to be will eventually die and disintegrate in the upper regions of the uterine horns at an early stage of their unsuccessful life. The mechanism whereby all but one of the fertilized eggs are eliminated and the reason why only one egg in each horn can continue its development have been considered before (van der Horst and Gillman, '41). Suffice to say at this juncture that there is only one very limited area in each horn of the uterus where implantation can take place and only the one fortunate egg that reaches this place can continue its normal development. Simultaneously with the arrival of this one embryo at the implantation site a strong contraction of the circular musculature of the uterus traps the others in the upper regions of the uterine horn, where soon they disintegrate. But even if some of the foremost in the long procession of descending eggs escape this fate and reach the lower part of the uterus, they will find the place occupied and their fate is not other than that of the eggs trapped in the upper regions of the horn.

The unfortunate eggs, doomed to an early death, may not even segment at all. Others develop up to the 2- or 4-celled stage when by the pressure of the contracted uterine wall the blastomeres are torn asunder. A few of the more vigorous ones, however, continue the unequal and hopeless struggle for some time. It is our intention here to record the history of these doomed embryos before they ultimately disintegrate and to compare their reaction with that which obtains in the normally developing embryo. This is of importance, because these abnormal embryos illustrate in a remarkable way the interaction existing between the endometrium and the ovum necessary for normal development.

The early developmental history of the surviving embryo has been fully recounted (van der Horst, '42, '44). Some of the impressive features, as far as they have a bearing on the present problem, may be recapitulated briefly.

Elephantulus is remarkable among mammals in that at an early stage of its development it forms a blastula, having about the same outward appearance as that of Amphioxus. The blastocoele was found to appear invariably when four blastomeres had been formed (fig. 1), but unless the implantation site is reached the embryo cannot develop beyond the 4-celled stage, neither does the blastula change in form and size. However, as soon as this place in the uterus has been reached, the embryo continues its development at once. This is shown by the fact that 4-celled embryos with or without blastocoele are of frequent occurrence in our collection of gravid uteri and they may be found anywhere in the Fallopian tube or uterine horn. It is unusual, however, to find a 4-celled embryo at the implantation site, since once it has arrived there it immediately continues its development. On the other hand, embryos with eight or more blastomeres were never found, except at the implantation site which shows that development beyond the 4-celled blastula can only proceed at the implantation site. When the blastomeres increase in number the blastocoele enlarges correspondingly, but the embryo remains a blastula with a single layer of cells until at least 100 cells have been formed. It is only then that the next developmental stage is initiated.

At the moment the one fortunate egg reaches the implantation site, not infrequently a few more eggs are in the same stage of development, i.e. they are young 4-celled blastulas (fig. 1). The four blastomeres of these embryos do not divide any further. These embryos remain in the 4-celled stage, but the secretion of fluid between the four blastomeres continues unimpeded until finally large distended vesicles, lined by very much flattened cells, are formed. Such a ballooned 4-celled blastula becomes larger than a normal blastula consisting of over 100 cells.

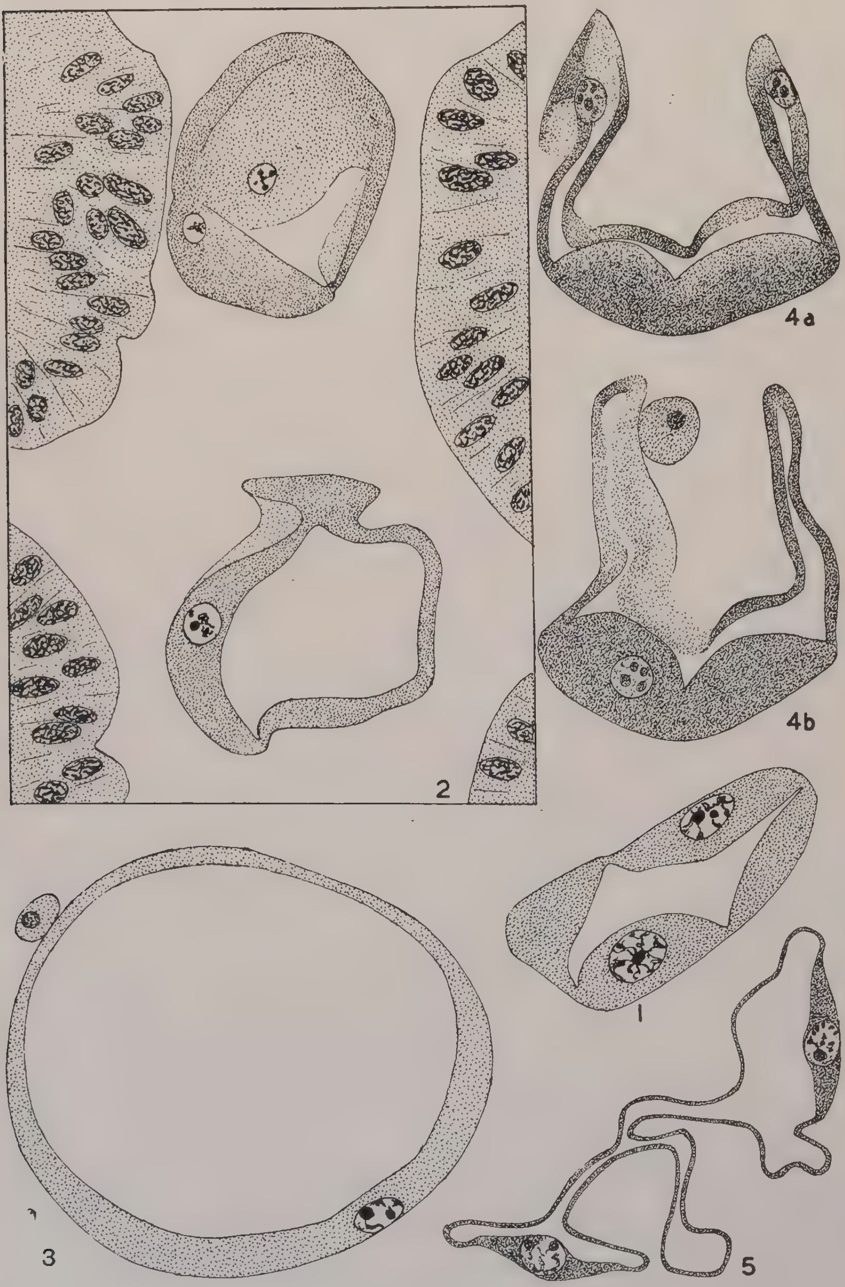
The extraordinary distention of the blastula and the modification in its structure may be appreciated from an examination of figures 1 to 5. A normal 4-celled embryo newly arrived at the implantation site, is depicted in figure 1. The four blastomeres are equal in size and form and in the center they are separated from each other by a small blastocoele. In the two 4-celled blastulas, lying in the upper uterus (fig. 2) and not destined to survive, the blastocoele is already enlarged; in their peripheral part the blastomeres are attenuated, but in the central part, containing the nucleus, the cytoplasm is much more abundant and here the wall of this blastula is much thicker. A section of a large swollen blastula with its attached polar body is portrayed in figure 3; the lining cells are extremely compressed by the pressure generated in the blastocoele. In figure 4 two sections of another swollen blastula are shown. This specimen is remarkable because only two of the blastomeres are attenuated, while the other two have retained their normal appearance. This might at first be taken as an indication of polarity, but as this is the only instance in at least a hundred blastulas so far examined, it is unlikely that much importance can be attached to this unequal development of the blastomeres.

These distended blastulas are nearly always collapsed in fixed preparations as shown in figure 4. A few regular spheres, like that in figure 3, where found in only one of our series. This collapse might be due to the injurious effects on such a delicate structure of fixation and dehydration. It may be presumed that normally these distended blastulas finally burst and then collapse; if this does occur it would be then impossible to identify with any certainty the remains of these cells as they cannot be distinguished from other debris lying in the uterine cavity.

In a few instances a moderately distended 4-celled blastula was found at the implantation site. In these rare cases it might be presumed that they had arrived rather late, so that they had accumulated already more than the normal amount of fluid in the blastocoele before coming under the influence of the implantation site. These distended blastulas might still have developed into normal embryos.

The 4-celled blastula shown in figure 5 is the largest in our collection. The unusual feature in this case was that it was lying in a large embryo chamber at the implantation site. In this instance the uterus also showed an abnormal reaction in that there was an excessive oedema in the endometrium similar to that depicted in figure 1 of our previous paper (van der Horst and Gillman, '42 b).

All figures are 500 times enlarged; the explanation is given in the text.



Figures 1 to 5

COMMENTS

We have seen that normally the embryo cannot develop beyond the stage of a small 4-celled blastula unless it arrives at the implantation site. But once it is lodged there it immediately proceeds to develop actively. From this it appears that the embryo is influenced in some manner by this small area of the uterus. On the other hand, the arrival of the embryo at the implantation site initiates a series of reactions confined to this small part of the endometrium (van der Horst and Gillman, '42 a). There appears to exist a mutual interaction between the uterus and the embryo, although direct contact between them is only established at a later period when actual implantation takes place.

During this period pre-implantation abortion may occur or the embryo may die prematurely (van der Horst and Gillman, '42 b). If this happens the reactions of the endometrium, that had set in already at the moment of the elimination of the embryo, continue but the subsequent reactions that would normally have followed do not make their appearance. Moreover, the reactions already induced continue in a more intense and exaggerated way, as they are no longer checked by subsequent reactions.

But the delicate mechanism of the interaction between uterus and embryo may be disturbed the other way round. This can only happen when a healthy and vigorous embryo is prevented from reaching the implantation site and thereby does not come under the influence emanating from that site. In this latter eventuality we see that the embryo behaves in a way similar to that of the uterus deprived of the embryo. The formation of fluid in the blastocoele had started already and this reaction of the embryo continues unchecked. On the other hand, further multiplication of the embryonic cells is prevented. The result is that the enormously distended, 4-celled blastulas are formed as already described.

SUMMARY

When in Elephantulus normal and fertilized eggs cannot reach the implantation site and thereby are excluded from the influence emanating from that site, they cannot develop beyond the 4-celled stage. However, the secretion of fluid in the blastocoele, a reaction already set in motion before the embryo reaches this site, continues unchecked, leading to a greatly distended, 4-celled blastula. The blastula is indispensable for the normal development of the decidual reaction at the implantation site in the uterus and the implantation site in turn is necessary for the normal development of the embryo.

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FAT EXCRETION IN THE GUINEA PIG KIDNEY

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TWO FIGURES

INTRODUCTION

Recent studies on the behavior of differentiated body cells toward fat, suggested a physical rather than chemical explanation for the movements of lipoids in and out of cells. This applied to sterols and carotenes found in cod liver oil, which are not saponifiable, and also to neutral fats.

Evidence has accumulated for particulate lipoid passage through the cell membrane, in the intestine (Munk, 1884); in the kidney (Wegelin, '13; Modell, '33); in the amoeba (Chambers and Kopac, '37a and b); in the adrenal (Zwemer, Wotton and Norkus, '38) and the liver and intestine (Wotton and Zwemer, '39). Observations suggesting particulate secretion by the adrenal cortex cells (Singer and Zwemer, '34) and by the mammary gland cells (Grynfeltt, '37) led us to study again the conditions of particulate fat excretion by the kidneys. Because of the differential staining and other properties of fat, histologic preparations of a fairly permanent nature can be prepared for observation of renal activity.

MATERIAL AND METHODS

Guinea pigs were chosen for this series of experiments, since their normal kidneys contain little or no visible fat. In this respect they differ greatly from the cat kidneys (Hartley, '07; Mottram, '16; MacMahon and Zwemer, '29; Modell, '33) which normally have numerous fat droplets in the proximal convoluted tubules.

Sixteen animals were used. They were in good health and weighed over 400 gm. each. Test meals of 6 to 10 cc. of cod liver oil saturated with Sudan IV, were placed in their stomachs by means of a very small diameter rubber catheter attached to a 10-cc. syringe filled with the oil. Animals were sacrificed from 1½ to 5 hours after feeding, at ½-hour intervals as indicated in table 1. Illuminating gas was employed as the lethal agent to avoid possible artifacts through the fat solvent action of ether or chloroform.

Kidneys were removed at autopsy and fixed in 10% pyridine formalin (Burke, '36). After fixation suitable blocks of tissue were cut and infiltrated with gelatin and sectioned on the freezing microtome after the method introduced by Zwemer ('33). Some sections were stained with Sudan IV to intensify the color already present in the fat, and counterstained with Harris hematoxylin, or iron hematoxylin. The latter demonstrated the mitochondria in many of the cells.

TABLE 1

Number of guinea pigs and time sacrificed.

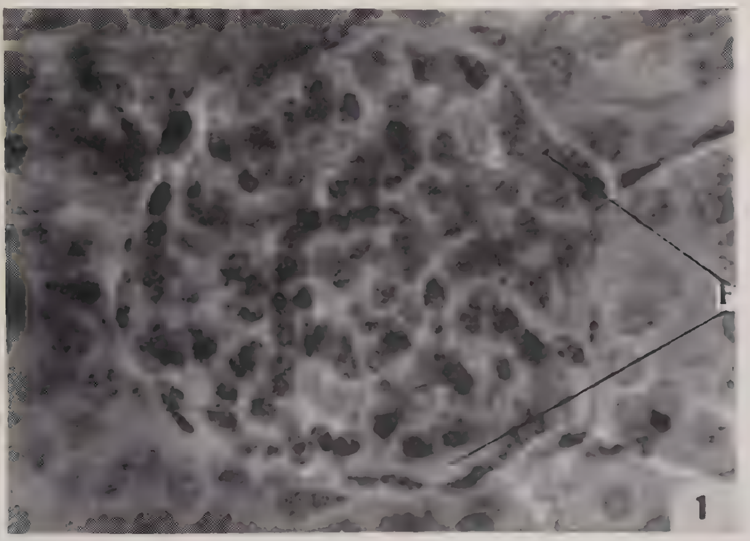
Hour after test meal	1½	2	2½	3	3½	4	4½	5
Oil fed	1	1	1	2	3	2	1	2
Control	..	..	..	1	1	..	..	1

HISTOLOGICAL OBSERVATIONS ON RENAL FAT DURING LIPEMIA

Fat in visible form was found to be excreted by the kidneys; the maximum amount being passed out in from 3 to 5 hours after feeding by stomach tube. On slicing open whole fixed kidneys, their cut surfaces showed fine red dots and short red striations in the region of the cortex, and longer red striations in the pyramids, indicating the presence of stained fat in the tissues. The stained fat, when present in the calyces, contrasted with the perirenal and hilar fat which was white in color. In those cases in which the guinea pigs urinated, colored fat floated on the urine.

Microscopic observation revealed red fat in the nephron. It was seen in the capillaries of the rete within Bowman's capsule, in the space of Bowman (fig. 1), in the lumen of the proximal convoluted tubule, in the cells of the convoluted tubule, in the small vasa efferentia surrounding the proximal tubules, and in the lumen of the loop of Henle. Most of the fat was seen in particulate form of large and small droplets but frequently hourglass drops (Wotton and Zwemer, '39) were observed where one bulb, situated in the lumen, was connected across a cell membrane by a fine strand to another bulb within that cell. Such forms were observed also where a globule of lipoid lay between two endothelial cells of a blood vessel, giving a characteristic picture of diapedesis. In the parenchymal cells of the proximal convoluted tubule, fat in a finely emulsified state lay in streams between the mitochondria which were parallel to the physiological axis of the cell (Pollister, '32 and '41).

Droplets of red stained fat were also seen in the lumina of the collecting ducts, in the cells of the duct walls (fig. 2), in the connective tissue surrounding these ducts, and in the capillaries in their vicinity. Within these cells the drops remained large, and never were finely emulsified



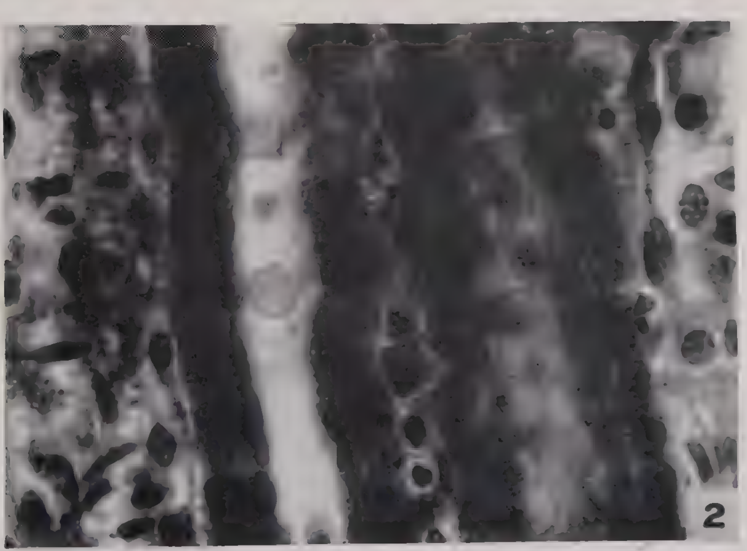


Fig. 1 In the glomerulus the fat (smooth gray in the photomicrograph) can be distinguished from the darker, chromatin-flecked nuclei. It is present in both the capillary rete and the capsular space.

Fig. 2 Fat can be seen in droplet form (not a cast) in the lumen of a collecting tubule and also passing through its walls.

as seen in the cells of the proximal tubules. In several instances stained fat was seen in the calyces.

An expression of the distribution of fat in the kidney as estimated by microscopic study of tissue sections is given in table 2.

The presence of fat in the urine usually is attributed to some pathological change in the kidney. However, one might expect a physiological excretion of fat into the urine by the kidney if large amounts are ingested. This must be a passage through cells of particulate fat for non-saponifiable lipoids, and even for neutral fats, unless one prefers the absurdly complicated explanation of a breakdown to water soluble products and resynthesis at each cell membrane.

TABLE 2

Relative stained fat distribution in four animals
4 = large amount; 3 = moderate; 2 = small amount; 1 = trace.

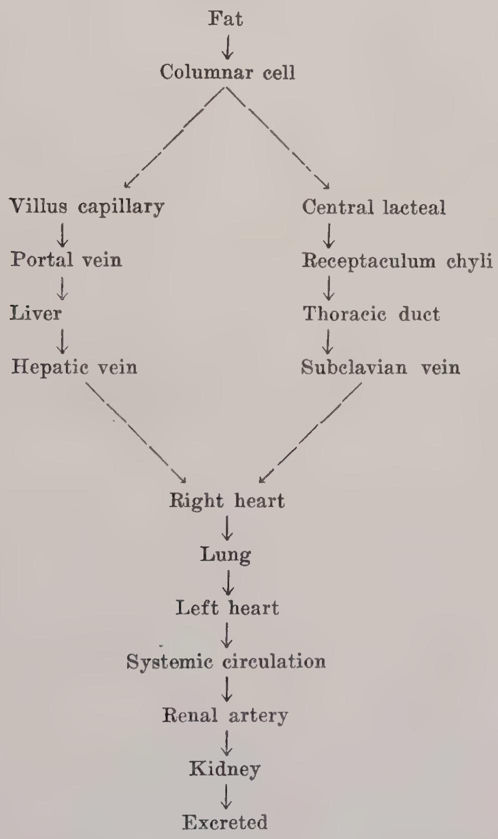
TIME	BODY WEIGHT	OIL GIVEN	GLOMERULUS	PROXIMAL CONV. TUBULE	CORTEX CAPILLARY	LOOP OF HENLE	COLLECTING DUCT	MEDULLARY CAPILLARY
	gm.	*cc.*						
3 hours	410	10	4	4	3	4	4	4
4 hours	450	8	1	2	2	1	4	4
4½ hours	410	6	1	1	1	3	4	3
5 hours	530	10	2	2	1	3	4	3

The kidney is one of the main organs for homeostasis of the blood. Its action, according to present thought (Best and Taylor, '39), is to retain the plasma proteins in the glomerular filter, and to reabsorb in the tubules, essential substances spilled out in the filtrate. It would appear that the nephron behaves toward particulate fat in the blood stream as it does toward other non-protein blood constituents.

When lipoid is absorbed from the alimentary canal it travels by two routes. The major portion (60–70%, Bodansky, '38) goes by the lymphatics, through the thoracic duct into the left subclavian vein, and thus into the pulmonary circulation. It passes through the lung capillaries, where little seems to happen to the fat except some slight phagocytosis by the dust cells, before reaching the systematic circulation. The remainder reaches the liver through the portal circulation. These routes of fat transportation are outlined in chart 1. According to recent work by Frazer ('43), the two routes may also be qualitatively different.

Fat transposition in visible droplet form from the glomerular capillaries into the space of Bowman is accomplished by diapedesis, between the overlying endothelial cells. That large fat globules can stream out while red blood cells are retained can be accounted for by the much greater plasticity of the lipoid. The visceral layer of Bowman's capsule seems to offer no barrier; in the adult it is believed to be a syncytium (Sobotta-Piersol, '30). We have in our previous studies observed this behavior of lipoid; it is probably governed by the same forces controlling transudation of fluids from capillaries into the tissue space.

CHART 1


Fat
Columnar cell
Villus capillary
Portal vein
Liver
Hepatic vein
Central lacteal
Receptaculum chyli
Thoracic duct
Subclavian vein
Right heart
Lung
Left heart
Systemic circulation
Renal artery
Kidney
Excreted

Due to the division of the arcuate artery, fat is carried to the cortex by the interlobular arteries, reaching the glomeruli by the vasa afferentia; and at the same time it is also transported by the arteria recta down into the pyramids. This accounts for the dual distribution of lipids in the kidney. The passage of stained fat through the epithelial cells of the collecting ducts is identical with that observed by us for the columnar epithelium of the small intestine and the paryenchymal cells of the liver. That drops are extruded by the columnar cells of the ducts is suggested by a second drop pushing into one already in the lumen (fig. 2). Also it will be noted that capillaries run in direct contact with the bases of these cells affording ample opportunity for the transfer of droplets.

In the kidney the hydrostatic forces set up by the pressure of the blood stream direct the movement of substances from the capillaries into the adjacent cells and tissue spaces. In the intestine the peristaltic waves and the individual movements of the villi set up stresses which cause the transition of substances from the lumen through the cells into the capillaries and lymphatics. It is our belief that mechanical forces plus surface tension control the direction of fat droplet transposition by the columnar absorbing cells of the small intestine and the columnar cells of the urinary collecting ducts of the kidney. The movement of fat in visible particulate form into and out of the body is controlled by these physical principles.

The appearance of fat in the cells of the proximal convoluted tubules is quite different, suggesting another mechanism of fat transport. These cells must do work to remove the droplets from the glomerular filtrate. The globules pass in at the distal or lumen border and leave at the capillary margin of the cells in the usual hourglass forms. But passage through the cells is accomplished by streaming between the mitochondria in a finely emulsified phase. Similar emulsification of large drops of fat absorbed by liver cells has been observed by us. Such a change in form indicates protoplasmic activity on the fat.

Pollister ('32 and '41) has shown that mitochondria and long chain protein molecules orient their long axes parallel to the cell currents. From this it is to be expected that in specifically polarized cells like the ones of the proximal convoluted tubules substances would be transported along the line of cell currents.

Marshall and Vickers ('23) accounted for their observations on kidney function on the basis of tubular excretion. Perhaps other substances in the blood, present in great excess, may be spilled out like fat through the epithelium of the collecting ducts.

It has been suggested that feeding stained fat would result in a coloring of fat already present in the body. This would reduce the intensity of the color, which we have not observed. Also, in some places there would be more of a transfer than in others resulting in a mottling effect. Gage and Fish ('23) were able to build up concentric layers of colored fat in the yolk of hen's eggs by alternate feeding of dyed fat. However, during the 5 hours of the present experiments, the depot fat remained white.

SUMMARY

The ability of mammalian kidneys normally to excrete fat in visible droplet form has been investigated.

Guinea pigs fed test meals of cod liver oil stained with Sudan IV, were sacrificed at selected intervals. Gelatin-embedded frozen sections were then made of the formalin-fixed kidneys. Our data indicates that fat is both excreted and reabsorbed. The two factors of amount of fat fed and time elapsed after feeding are reflected in the histological findings.

If our interpretations be correct, one can infer a kidney mechanism for fat similar to that regulating the excretion and reabsoption of electrolytes, glucose and other threshold substances.

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THE PRODUCTION OF MULTIPOLAR MITOSES IN NORMAL SOMATIC CELLS OF EMBRYONIC CHICK TISSUE GROWN IN VITRO

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TWO PLATES (TEN FIGURES)

The somatic cell mutation theory as formulated by Boveri ('29) remains today as a satisfactory working hypothesis for investigations upon the origin of maligancy. In fact a committee appointed by the Surgeon General to report upon the fundamental aspects of the cancer problem in 1938 made the following statement as one of their tentative proposals: "The new property (i.e. malignancy) of the cell appears to develop suddenly, becomes a fixed character and is transmitted to its descendents. It gives evidence of being a somatic mutation."

The mechanism of action whereby somatic mutation may be produced experimentally is as yet unknown. The site of action for such a mechanism is generally believed to be the chromatin material of the cell nucleus. Various physical as well as chemical agents have been found by numerous investigators to disturb the normal behavior of chromatin during cell division. Thus, growing cells following exposure to radium, x-ray, heat, cold, carcinogenic and related substances have been observed to exhibit pycnotic mitoses, fragmented and lagging chromosomes as well as precocious splitting of chromosomes (Hertwig, O., and R., 1887; Politzer, '34; Lewis, M. R., '35; Creech, '39; Tennent, '42; Stilwell, '40, '42, '43 and many others). Yet the statement of Kemp and Juul ('30) regarding the results of experimental work in this field of investigation remains pertinent today. They point out that "there is a complete absence of certain changes that are characteristic of anaplasia of cancer tissue, such as multipolar mitoses, very large mitoses and marked variations of chromosome count."

The present report describes the appearance of numerous multipolar mitoses and giant cells produced in tissue cultures prepared from 8-day chick hearts. Four series of cultures were made in the spring of 1943 and five series in 1944. A preliminary statement concerning the results obtained in 1943 has already appeared (Stilwell, '43).

MATERIAL AND METHODS

Nine series of cultures of 8-day chick hearts were prepared by the usual hanging drop technique. A total of thirty-two to thirty-four chick hearts were used in the nine series of cultures. The temperature for incubation was constant at a given point in the incubator but varied from 42± °C. at the back to 37½°C. at the front. The heating units of the incubator consisted of two electric light bulbs (carbon). Light of very low intensity was therefore emitted intermittently during the entire period of incubation. All cultures placed in the back half of the incubator were killed.

The tabulation on page 117 gives data regarding procedure and culture media used in the nine series of experiments.

CYTOLOGICAL EXAMINATION OF CULTURES

The microscopic examination of cultures was carried out with a 2-mm. apochromatic oil immersion lens (N. A. 1.3) and a 12 × compensating ocular. A considerable number of cultures were examined cell by cell for atypical division figures and mitotic counts were made in these instances. As this procedure is very tedious and time consuming many of the cultures were examined with high dry and immersion oil was applied only when an atypical mitotic configuration was encountered. The unusual size of cells exhibiting multipolar mitoses also served as an aid to their disclosure in many cases.

Group 1. Cultures not transferred, series 2, cultures 1, 4, 14, 19, 23, 24, 26, and 31; series 3, cultures 1, 2, and 22.

Chart 1 shows the location in the incubator of the eleven cultures comprising this group. Four cultures were fixed after 48 hours in vitro, three after 6 days in vitro and four after 7 days in vitro. Microscopic examination of these cultures reveals two types of cellular degeneration. The presence of large numbers of rounded-up dead or dying cells is indicative of degeneration caused by high temperature. Cultures placed in the back half of the incubator exhibit this type of degeneration. In cultures of 6–7 days incubation grown at compatible temperatures mitotic figures are rare and the peripheral cells are fat laden. These findings indicate a lack of nutritive material in the medium.

Culture 1, series 2 was fixed at the end of 48 hours in vitro. Among the 400± cells undergoing division in this culture three multipolar mitoses are present. Two of these are atypical while the third exhibits two spindles with one pole in common.

Group 2. Cultures transferred; fixed from 2–6 hours after transfer; series 2, cultures 5, 6, 7, 8, 9, 10, 15, 16, 17, and 18.

SERIES	DATE OF EXPLANTATION	NATURE OF EMBRYONIC JUICE	PLASMA [1]	DATE OF TRANSFER	NATURE OF EMBRYONIC JUICE	PLASMA	DATE OF FIXATION [2]
1 24 cultures. (12 killed by excessive heat)	4/7/43	2 drops fresh embryonic juice from 11- and 8-day chick embryos (dil. 1: 1 with Tyrode)	1 drop of fowl plasma (dil. 1:1 with Tyrode)	4/13/43 (12 cultures)	2 drops of embryonic juice stored in refrigerator from 4/7/43 (dil. 1: 1 with Tyrode)	1 drop of fowl plasma (dil. 1: 1 with Tyrode)	4/14/43 (7 days in vitro)
2 32 cultures	4/21/43	Same as above	Same as above	4/27/43	Same as above	Same as above	4/27/43 and 4/28/43 (7 and 8 days in vitro)
3 24 cultures	4/28/43	Same as above	Same as above	5/5/43	2 drops of fresh embryonic juice from 11- and 8-day chick embryos	Same as above	5/6/43 (8 days in vitro)
4 12 cultures	5/5/43	Same as above	Same as above	5/12/43	2 drops of embryonic juice stored in refrigerator from 5/5/43 (dil. 1: 1 with Tyrode)	Same as above	5/13/43 (8 days in vitro)
5 16 cultures	4/28/43	2 drops of fresh embryonic juice from 3 (8-day) and 2 (11-day) chick embryos (dil. 1: 1 with Tyrode)	1 drop of fowl plasma (dil. 1:1 with Tyrode)	5/5/44	2 drops of embryonic juice from 4 (8-day) and 1 (11-day) chick embryos (dil. 1: 1 with Tyrode)	1 drop of fowl plasma (dil. 1: 1 with Tyrode)	5/6/44 (8 days in vitro)
6 16 cultures	5/5/44	2 drops of fresh embryonic juice from 4 (8-day) and 1 (11-day chick embryos (dil. 1: 1 with Tyrode)	Same as above	5/12/44	Same as above	Same as above	5/13/44 (8 days in vitro)
7 * 16 cultures	5/12/44	Same as above	Same as above	5/19/44	Same as above	Same as above	5/20/44 (8 days in vitro)
8 * 16 cultures	5/19/44	Same as above	Same as above	5/26/44	2 drops of embryonic juice from 5 (8-day) and 1 (11-day) chick embryos (dil. 1: 1 with Tyrode)	Same as above	5/27/44 (8 days in vitro)
9 * 16 cultures	5/26/44	2 drops of fresh embryonic juice from 5 (8-day) and 1 (11-day) chick embryos (dil. 1: 1 with Tyrode)	Same as above	6/2/44	2 drops of embryonic juice stored in refrigerator from 5/26/44 (dil. 1: 1 with Tyrode)	Same as above	Cultures 1–12 inc. 6/3/44 and cultures 13–16 inc. 6/4/44 (8 and 9 days in vitro)

[1] The plasma used on all cultures of series 1–4 inclusive was obtained from a fowl on 3/15/43 and was stored in a refrigerator until used.

The plasma used on all cultures of series 5–9 inclusive was obtained from a fowl (not from same stock as above) on 4/18/44 and stored in a refrigerator until used.

[2] All cultures were fixed according to the method of Champy and stained by Heidenhain's iron haematoxylin method as modified by Patton and Wigoder ('39). In toto mounts were made of all cultures.

* Even numbered cultures were wrapped in black paper.

CHART 1

Location of cultures in incubator.

Series 2

									42 ± °C. ↑	
1^{x}	5	9	15^{d}	19^{d}	23	27				back half of incubator
2	6	9^{x}	12^{x}	16	20	24	28	31	↓ 37½ °C.	front half of incubator
3	7	10^{x}	13^{x}	17	21	25	29	32^{x}		
4	8	11*	14	18	22^{x}	26	30			

Key

^{x} multipolar mitoses present
^{d} complete degeneration
* accidently destroyed

Series 3

								back half
								front half
1	4^{d}	7^{d}	10^{x}	13^{d}	16	19^{d}	22^{d}	
2	5^{x}	8^{d}	11^{x}	14^{d}	17	20^{d}	23^{x}	
3*	6^{d}	9^{d}	12	15	18^{x}	21	24	

Series 4

				back half
				front half
1^{x}	4^{x}	7	10^{x}	
2^{x}	5^{x}	8^{x}	11^{x}	
3	6^{d}	9^{d}	12^{x}	

Series 7

				13^{d}	back half
					front half
1^{d}	4^{d}	7^{d}	10^{d}	14	
2^{d}	5	8^{d}	11	15	
3	6^{d}	9^{d}	12^{x}	16^{d}	

Series 5

				13	back half
					front half
1	4	7	10	14	
2	5	8	11	15^{x}	
3	6	9	12	16	

Series 8

				13	back half
					front half
1	4^{x}	7	10	14^{x}	
2	5^{x}	8^{x}	11	15^{d}	
3*	6	9^{d}	12^{x}	16	

Series 6

				13	back half
					front half
1	4	7	10	14	
2	5	8	11^{x}	15	
3	6	9	12^{x}	16	

Series 9

				13	back half
					front half
1	4	7	10	14	
2^{x}	5	8	11	15	
3	6	9^{x}	12	16	

Chart 1 shows the location in the incubator of the ten cultures comprising this group. Four cultures were fixed 2 hours after transfer to fresh medium; three cultures at 3¼ hours and four cultures at 6 hours.

The cytological picture presented by these cultures is for the most part similar to that found in cultures of group 1. In cultures 5 and 7 marked liquefaction of the medium had taken place prior to transfer. Culture 15 degenerated completely. The total number of mitoses per culture was recorded in three instances. Less than 10 mitoses are present in each of two cultures (17 and 18) while there are 98 in the third culture (10).

Tripolar mitoses are present in cultures 9 and 10 which were fixed 3¼ hours following transfer. In three instances the cell exhibiting this mode of division is an extremely large cell, comparable in size to that of figure 10. A few cells with lobulated prophasic nuclei were encountered. One cell (culture 17) displays distinctly double chromosomes passing to the poles of a bipolar spindle.

Group 3. Cultures transferred; fixed 23–25 hours after transfer.

Chart 1 shows the location in the incubator of the 147 cultures comprising this group. Six cultures of this group were accidently destroyed. Microscopic examination of the remaining 141 cultures revealed marked degeneration in 43 cultures (30%). Therefore only 96 cultures exhibit a vigorous growth in which frequent division figures are present. Multipolar mitoses have been found in 38 cultures (38.7%). It is significant that this type of cell division is present in one or more cultures of each series. Reference to chart 1 will show that multipolar mitoses are present in a greater number of cultures belonging to series 1, 2, 3, 4, and 8 than are present in cultures of series 5, 6, 7, and 9. Since the cultures of the former (series 1, 2, 3, 4, and 8) have received more intensive study than the latter series it seems reasonable to presume that further study would reveal additional cultures with multipolar mitoses in these series. It is noteworthy that 80% of the healthy cultures of series 1 and 72.7% of series 4 show multipolar figures. The culture exhibiting the greatest number of multipolar division figures is culture 10 of series 1. Of the 780± mitoses present in this culture 14 are multipolar (1.8%). Binucleated cells are present but are not of frequent occurrence.

The percentage (30%) of cultures in this group showing degeneration is relatively high, but this may be explained if the following facts are taken into consideration.

All cultures except those of series 1 and 2 were transferred on the seventh day of incubation. More frequent renewal of nutrient media is recommended for the promotion of growth of fibroblasts. It is therefore probable that in a number of instances the degenerative changes in the fibroblasts had progressed to a point which precluded recovery under the existing conditions. A second factor causing degeneration was excessive temperature. All cultures placed in the back half of the incubator exhibited heat degeneration. A third cause of degeneration was a modification of procedure. The main explant was cut away from the central part of eight cultures of series 5 and 6, thus releasing tensional forces conducive to progressive growth. Recovery from this disturbance had not taken place by the time these cultures were fixed.

The location of cultures comprising series 1 is not shown on chart 1. This series consisted of twenty-four cultures, twelve of which were placed in the back half of the incubator and twelve were located in the front half. All cultures of the former group died. The exact location of the cultures placed in the front half of the incubator was not recorded.

Group 4. Cultures transferred; fixed 48 hours after transfer; series 9, cultures 13, 14, 15, and 16.

Chart 1 shows the location in the incubator of the four cultures comprising this group. No significant findings as a result of the longer period of cultivation are evident upon the microscopic examination of these cultures. A tripolar mitosis (late telophase) is present in culture 16. Cultures 13 and 15 show marked degeneration.

CYTOLOGY OF MULTIPOLAR CELLS

In tissue cultures of embryonic chick heart muscle the predominating types of cells to be found in an 8-day culture are fibroblasts, macrophages and muscle cells. The cytological examination of the cultures which exhibit a vigorous growth reveals an abundance of dividing cells (more than 1,000 in culture 4, series 4). Not all of the dividing cells are normal as in numerous instances rounded-up cells with pycnotic masses of chromatin or chromosomes are present. These are abortive mitoses which could lead only to cell degeneration.

In the marginal growth of cultures of series 1 cells of extraordinary size (fig. 10) were at once noted. In many cases these giant cells were found to be undergoing multipolar mitosis. Subsequent study of the nine series of cultures revealed numerous tripolar and tetrapolar division figures in medium sized cells as well as in giant cells. Although no experiments have been made to establish the identity of the cells exhibiting multipolar mitosis they appear to be fibroblastic in character.

They are easily distinguishable from macrophages and heart muscle cells.

It should be emphasized that all phases of the mitotic cycle (except prophase) have been seen in multipolar cells. It is also significant that there is considerable variability in the configurations presented by these abnormal mitoses which in itself is suggestive of conditions found in certain neoplasms. Although the majority of the multipolar division figures are triasters a number of tetrasters have been encountered. In culture 8, series 8 a tetrapolar division figure with a fifth (diagonal) spindle is present. In addition to these unusual mitoses a few (5–6) bipolar metaphases with extremely broad spindles bearing an excessive number of chromosomes are present. No attempt to count the chromosomes was made owing to their small size and compact arrangement on the spindle. Since a detailed description of a large number of individual cells exhibiting multipolar mitosis would serve no real purpose, only a few cells which represent types of multipolar configurations will be described.

In culture 5, series 1 a total of six tripolar mitoses is present (3 metaphases, 2 telophases and 1 reconstruction phase). Figure 1 is a tracing and photomicrograph of a small area from the marginal growth of this culture. Two tripolar cells are located within a single field (4-mm. objective). In the upper cell of the photomicrograph three metaphasic plates of chromosomes are clearly visible whereas in the lower dividing cell three telophasic masses of chromosomes are evident. It is regrettable that the photomicrograph fails to reveal the spindle substance of the metaphase with clarity. There are, however, two reasons for this. In the first place the cell is so rounded-up that the optical level revealing the chromosome masses with the greatest sharpness does not correspond with the level at which the spindle substance is most clearly visible. Secondly, the fixing fluid (Champy) is an acid free fixative. Acid free fixatives are not recommended for the best preservation of the achromatic figure of mitosis. It must be stressed at this point that under the microscope the spindle of this metaphase (and others as well) is plainly visible. There is no evidence of the destruction of spindle substance in the normal multipolar metaphasic division figures.

Figure 5 is a tracing and photomicrograph of a tripolar telophase from the same culture (culture 5, series 1). It is evident that the distribution of the chromatin material to the three poles is unequal in amount. A lagging chromosome, approaching the largest mass of chromatin is visible. An appreciation of the thickness of this cell is easily attained since the upper right chromatin mass is not in exact focus with the other two chromatin masses.

Figure 4 is a tracing and a photomicrograph of an early anaphase (tripolar) from culture 2, series 1. In this cell two spindles are of greater breadth and length than the third spindle. Asymmetrical tripolar figures such as this occur frequently. The chromosomes of this cell do not look normal since they appear to be fragmented or granular.

Figure 8 shows a cell from culture 4, series 1. Masses of chromatin material lie scattered in the cytoplasm without any trace of spindle substance. The fate of such cells is questionable. It may be that only cell degeneration results. On the other hand chromosome vesicles may be reconstituted from the scattered chromatin masses. A few cells have been found exhibiting chromosome vesiscles of variable sizes.

Figures 3 and 7 are tracings and photomicrographs from cultures 22 and 32 respectively of series 2. The cell of figure 3 exhibits three poles but only two spindles. The juxtaposition of a bipolar and tripolar metaphase in figure 7 is particularly vexing when considered in relation to any role which an exogenous agent alone might play in producing multipolarity. It should be noted that the tripolar cell is considerably larger than the normal bipolar cell.

An early tripolar anaphase from culture 23, series 3 is shown in figure 2.

From culture 10, series 4 a late telophase with division furrows of the cytosome is shown in figure 6. Cells with complete division of the cytosome into three daughter cells, each with a reconstructed nucleus, have been encountered in a number of cultures.

An example of one of the largest giant cells encountered, a tripolar metaphase, is shown in figure 10. This enormous cell is located about midway betwween the main explant and the periphery of the marginal growth of culture 10, series 1. The triaster of this cell consists of one very broad spindle bearing a very large number of chromosomes and two slender spindles with attached chromosomes. Attention is called to the fact that the magnification of this photomicrograph (fig. 10) is the same as that of photomicrographs 2–8 inclusive.

Figure 9 is a photomicrograph of a prophase from culture 32, series 2. It reveals a deeply notched condition of a relatively large nucleus. A number of large notched or lobed prophasic nuclei have been noted among the dividing cells of these cultures. It is not known whether these unusual configurations of prophases are of any significance in relation to multipolarity.

DISCUSSION

Owing to the sporadic nature of the phenomenon of multipolar cell division it has been extremely difficult for investigators to evaluate its significance in relation to either normal or neoplastic growth. Only a method whereby this type of cell division may be produced experimentally can possibly lead to tenable deductions regarding the potentialities of daughter cells.

In the present work significant numbers of multipolar dividing cells and giant cells have been produced in nine series of tissue cultures of 8-day embryonic chick hearts. Four series were made in 1943 and five series in 1944. It is well known that multipolar mitoses are of rare occurrence in cultures of normal embryonic chick material. Kemp ('30) found only a single tripolar mitosis among 10,000 dividing cells in tissue cultures of various organs of normal chick embryos and adult fowls. The numbers of multipolar mitotic figures (1–14) present in individual cultures of the present work equal or exceed the numbers which have been reported for cultures of malignant tissues. Goldschmidt and Fischer ('29) examining cultures of mouse carcinoma found only two triasters in 100 mitoses. Von Mollendorf ('40) reports upon mitotic counts of five cultures of a Brown-Pearce tumor metastasis. The highest number of multipolar cells in a single culture was 7. The variability of the multipolar configurations in the cells of the cultures under consideration is also noteworthy. It is as great as that shown by Lewis and Lewis ('32) in their study of tissue cultures prepared from the Walker rat sarcoma no. 338.

Whereas multipolar mitoses have been successfully produced in the nine series of cultures under discussion it is not known as yet what factors are instrumental in their production. In analyzing the experimental procedure certain elements come to our attention. It is known that the temperature of the incubator varied from 42±°C. at the back to 37½°C. at the front. The heating unit of the incubator consisted of two electric light bulbs (carbon) and therefore light of low intensity and long wave lengths was emitted intermittently during the entire incubation period. The age at which the cultures were transferred, i.e. 7 days (with the exception of series 1 and 2) may be significant. The possibility was also considered that a constitutent of the plasma, peculiar to an individual fowl, might prove to be a significant factor in evoking multipolartiy.

Up to the present time only two of the possible contributory factors have been ruled out. The plasma used in series 5, 6, 7, 8, and 9 was taken from a fowl of different stock than that used in the first four series. It thus seems apparent that no constituent or property of the plasma peculiar to an individual fowl is the causative agent for the appearance of multipolar division figures.

The possibility of a photodynamic action due to some constituent of the nutrient medium was also considered. The even numbered cultures of series 7, 8, and 9 were therefore wrapped in black paper during their entire incubation period. Reference to chart 1 will show that twice as many even as odd numbered cultures in these series exhibit multipolar cells. It may be concluded that photosensitivity of the cells is not a factor in the production of multipolarity in these experiments.

Since the early work of Hertwig (1887) and others the effect of abnormal temperatures upon the processes of growth have received intensive study. Variations in temperature alone have hitherto not been shown to produce unusual numbers of multipolar mitoses.

The possibility remains that a variation in temperature combined with additional factors may conceivably be responsible for evoking multipolarity. Without further experimentation it is only possible to suggest that the physiological state of the cells may in some way be a contributory factor in this phenomenon. After 7 days in vitro without transfer the fibroblasts of the marginal growth start to degenerate. Wilmer and Jacoby ('36) (working with cultures of fibroblasts) found that degeneration commences on the sixth day of in vitro life and that after 8 or 10 days there is no further increase by mitosis (i.e. without renewal of medium). Stilwell ('38) also pointed out that fibroblastic growth but not that of muscle cells was inhibited when cultures were transferred only every 7 days. It follows that in the present experiments a rich nutrient medium (embryonic extract of 8- and 11-day embryos) was added possibly at a crucial moment in the life of certain fibroblasts. It should also be borne in mind that Claude ('38) isolated a fraction of embryonic juice which in chemical composition is remarkably similar to a tumor producing fraction isolated from the Rous sarcoma. Claude raises the question "whether the substance found in normal chick embryo tissue may represent a precursor of the chicken tumor principle which would assume, under certain conditions, the self-perpetuating properties of the tumor agent."

Henshaw ('40) discusses the production of multipolar cleavage of eggs of Arbacia punctulata which had been exposed to heavy dosages of x-ray. His results are of basic significance in that he has shown how at least one mechanism (x-ray irradiation) may conceivably produce "cell death on the one hand and malignant growth on the other." It remains to be seen whether or not the results obtainable with fertilized eggs will prove applicable to somatic cells.

It is hoped that a rigid analysis of procedure and material will in time disclose the factor or combination of factors which evoke multipolarity in somatic cells under the conditions described here. It should also be possible to follow the fate of the daughter cells resulting from multipolar division in living cultures.

SUMMARY

It has been demonstrated that multipolar mitoses can be produced experimentally in normal somatic cells of embryonic chick heart tissue grown in vitro. Nine series of culures were made and examined microscopically for atypical division figures. Multipolar division figures are present in thirty-eight (38.7%) of the ninety-six cultures exhibiting a vigorous growth.

A description of the occurrence and cytology of the multipolar mitoses (triasters, tetrasters and aberrant forms) is given. Attention is called to the fact that both the number of multipolar division figures present and the variability in their configurations is suggestive of the conditions found in certain neoplasms. The technical procedure and the content of the culture medium are discussed in relation to the possible factor or combination of factors which may be instrumental in evoking multipolarity under the conditions of the experiments.

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PLATE 1

EXPLANATION OF FIGURES

Photomicrographs and outline tracings (facing). Reduction one-fifth.

1A and B Tracings and photomicrograph of cells from culture 5, series 1. The upper cell exhibits a tripolar (early anaphase) mitosis; the lower cell exhibits a tripolar (telophase) mitosis. × 1100.

2 Tracing and photomicrograph of cell from culture 23, series 3. Tripolar (early anaphase) mitosis with scattered chromosomes on two of the three spindles and a plate of chromosomes on the third spindle. × 1530.

3 Tracing and photomicrograph of cell from culture 32, series 2. Tripolar (early anaphase) mitosis showing three poles with only two spindles. Fat droplets shown in outline. × 1530.

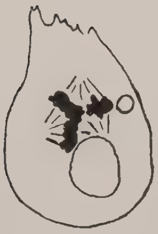
4 Tracing and photomicrograph of cell from culture 2, series 1. Tripolar (early anaphase) mitosis exhibiting unequal distribution of chromosomes on the three spindles. Chromosomes appear fragmented. × 1530.

5 Tracing and photomicrograph of cell from culture 10, series 4. Tripolar (late telophase) mitosis exhibiting blebs and division furrows of cytosome. × 1530.

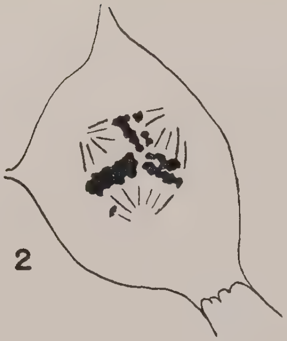
6 Tracing and photomicrograph of cell from culture 5, series 1. Tripolar (telophase) mitosis exhibiting telophasic ''asters'' of unequal size; a ''lagging'' chromosome lies near the largest aster. × 1530.

7 Tracing and photomicrograph of cells from culture 22, series 2. Bipolar and tripolar (metaphase) mitoses in contiguous cells. × 1530.

8 Tracing and photomicrograph of cell from culture 4, series 1. Chromosomes (without spindle substance) lie scattered in cytosome. × 1530.



1A



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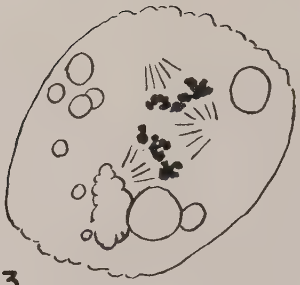

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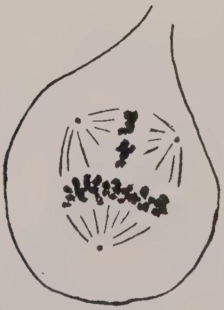

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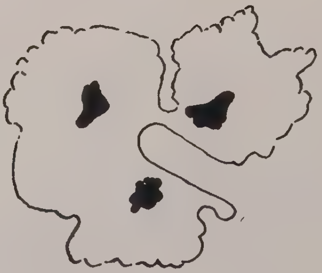

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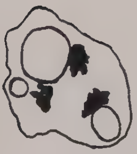

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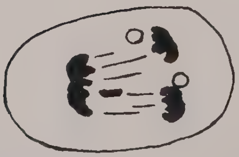

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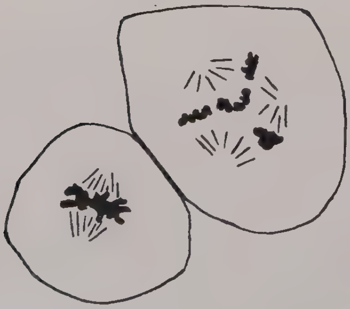

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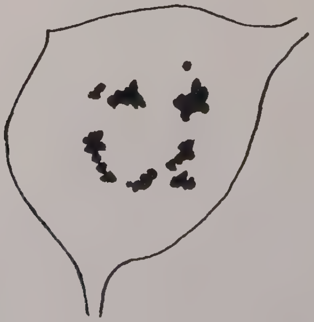

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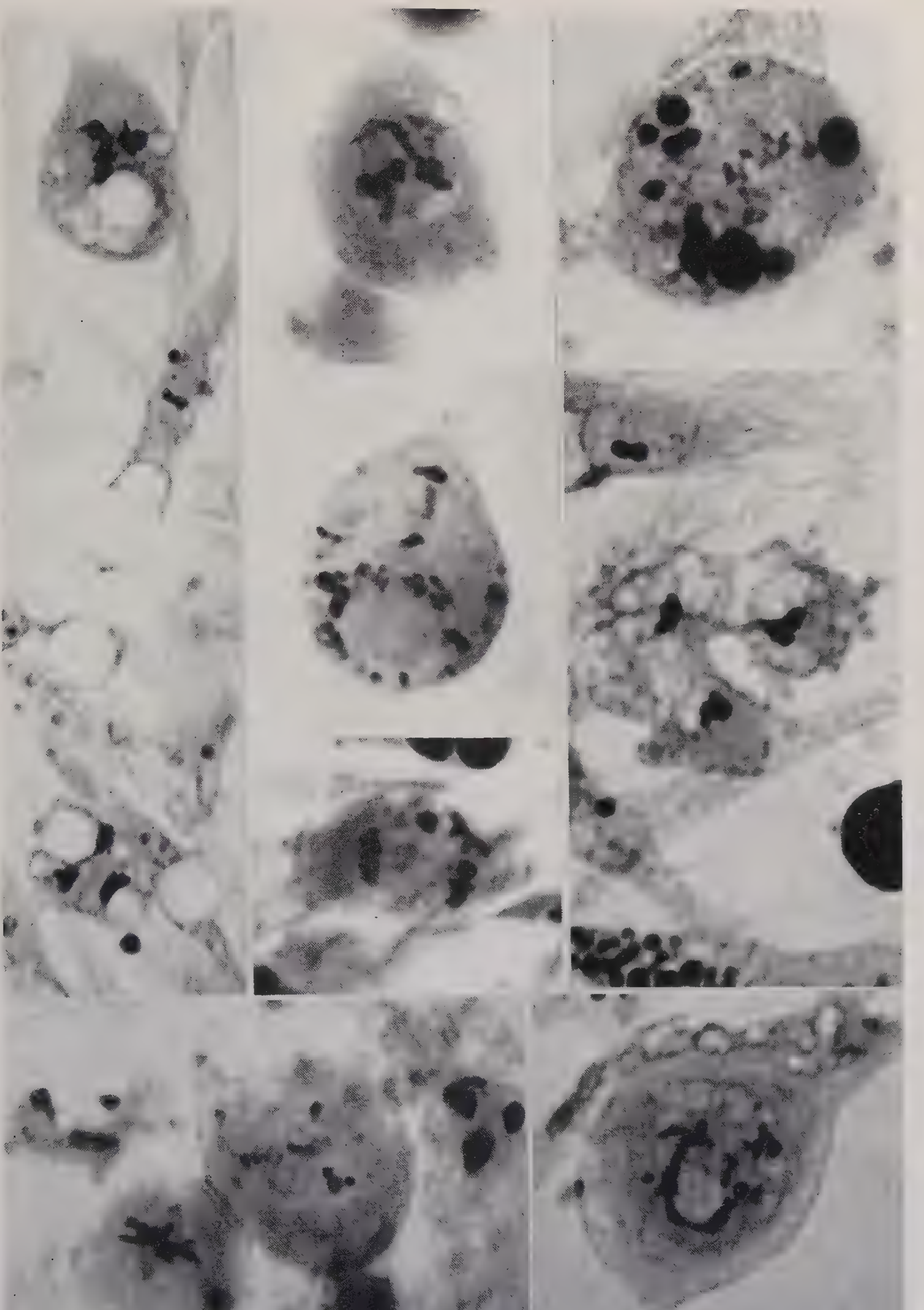


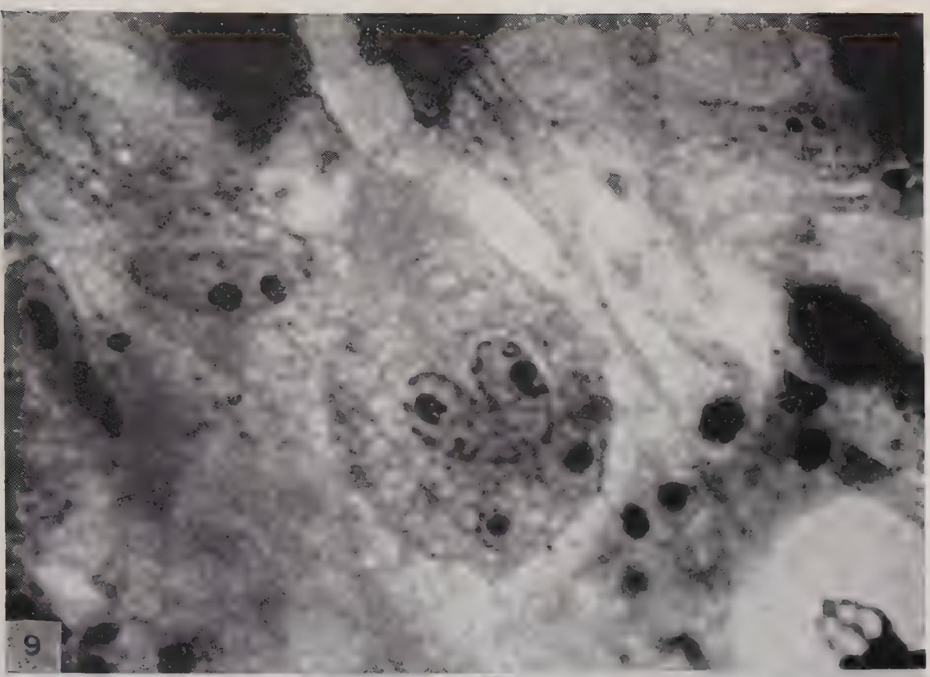
PLATE 2

EXPLANATION OF FIGURES

Photomicrographs magnified 1530 ×. Reduction one-fifth.

9 Photomicrograph of giant cell from culture 32, series 2. Note deeply notched configuration of prophasic nucleus.

10 Photomicrograph of giant cell from culture 10, series 1. Tripolar (metaphase) mitosis exhibiting one very broad spindle and two slender spindles. Compare cell with those of figure 7 (same magnification).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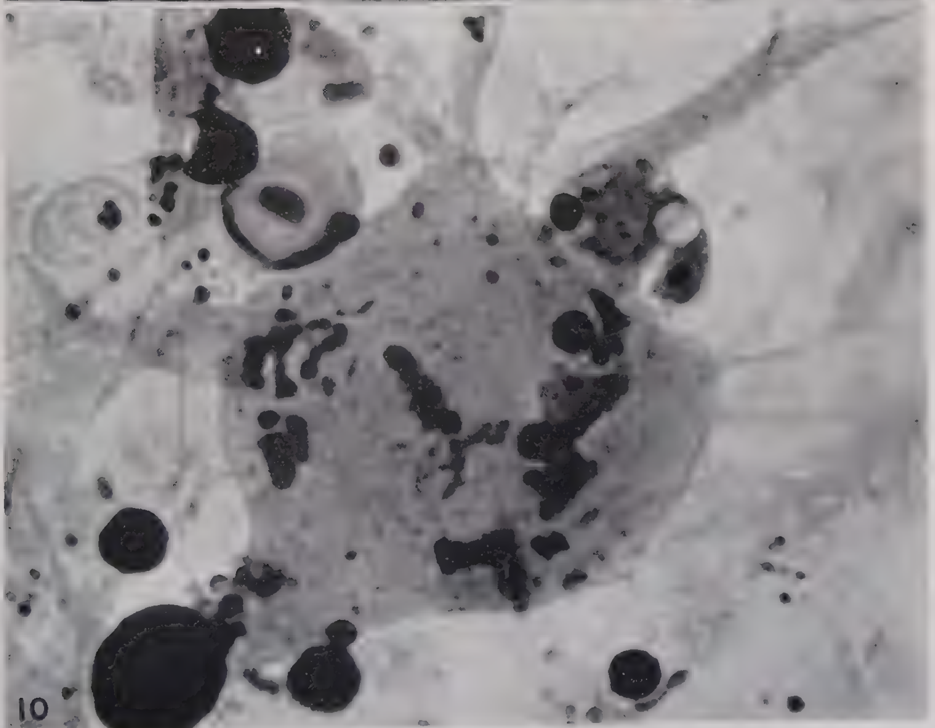
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MITOTIC ACTIVITY IN THE THYROID GLAND OF FEMALE RATS[1]

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INTRODUCTION

The rate of mitotic cell division in certain organs has been shown to vary with such factors as the phase of the estrous cycle, with age, and with the time of day. This paper is concerned with the extent to which these factors and especially the estrous cycle affect the rate of division in the thyroid gland of the female rat. By using the rat it will be possible to correlate the mitotic activity of the thyroid with that of other structures which have already been studied (Hunt, '40, '42, '43). Such a correlation is desirable since it would be helpful to know whether a factor acts specifically on one organ or on a group of related organs or whether it acts indirectly or not at all.

During the estrous cycle the mitotic activity varies markedly in a number of organs. In the rat these include the ovaries (Lane and Davis, '39), genital epithelia (Allen et al., '43), the hypophysis (Hunt, '42), and adrenal gland (Hunt, '40). Some organs, however, such as the intestine (Hunt, '42) are not affected and of those that are, not all have have an increased activity at the same time. The genital epithelia and the hypophysis have more mitoses at late estrus and the adrenal more at diestrus. It thus appears that during the cycle the increased mitotic activity in the various structures is due to more than one factor that act in sequence.

In order to evaluate the effect of the estrous cycle on mitotic activity it is necessary to know the extent to which other factors such as age and time of day affect the activity. In the hypophysis the increased activity at late estrus becomes markedly reduced as animals become older (Hunt, '43) so that an animal of 300 days of age does not have many more mitoses at late estrus than one of 100 days at diestrus. The time of day at which an animal is killed is likewise an important factor to be controlled, since in practically all organs where mitoses have been studied there is an increased activity at one or more periods of the day.

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[1] Aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research and the University of Alabama Research Fund.

Furthermore Blumenfeld ('42) has found that the periods of increased activity are not the same for all structures of the same animal and he concludes "that the factors which determine when and how often mitosis occurs act on or reside within the organ as a unit".

ACKNOWLEDGMENTS

I wish to thank Dr. C. M. Goss for supplying the rats from his colony and for his helpful criticisms in the preparation of the paper.

MATERIALS AND METHODS

The 131 thyroid glands examined for mitotic activity were from the same rats that supplied the hypophyses used in previous reports (Hunt, '42, '43). Tissue was fixed in Allen's B-15 fluid and cleared in dioxan after passing through alcohol from 10 to 70% in 10% steps. Sections were cut at 3 micra and every tenth section was mounted and stained according to Masson's procedure. Four or five sections from each gland were examined for mitotic figures. This represents an area approximating 12.5 sq. mm.

About half of the animals received colchicine 9 hours before being killed. By halting division at the metaphase during this period the mitotic count was approximately tripled and proved especially valuable in the counts from the glands of older animals and in those killed in diestrus where the mitotic figures were few and scattered. Animals receiving colchicine were, with fourteen exceptions, killed between 4 and 5 P.M. Those not receiving it were killed between 11 and 12 A.M.

The stages of the estrous cycle were determined by vaginal smears.

RESULTS

A definite correlation was found between the mitotic activity in the thyroid gland and the sexual cycle. In groups not receiving colchicine and of comparable age, killed during each of the five phases of the estrous cycle (groups 1–5, table 1), there was a significantly higher mitotic activity in the group killed in late estrus. Minimum activity was found during proestrus and diestrus and an intermediate activity occurred in early estrus and metestrus.

The variation at different phases was even more striking in the animals that received colchichine. Comparison of groups 6c, 7c, 9c, 12c, and 13c (table 1), which were of comparable ages, shows that animals killed in late estrus have by far the highest mitotic activity. The invariable low counts of animals killed in proestrus and diestrus in this series is considered very significant since the use of colchicine rules out the possibility of killing animals at a low ebb of mitotic activity that might rise a few hours later. The intermediate activity in the early estrous phase (group 7c) is not apparent in the animals that received colchichine, because they were killed in the early part of the phase whereas in the comparable group without colchicine some were killed in the late part.

TABLE 1

Mitotic activity in the thyroid gland of groups of female rats with and without colchicine.

GROUP [2]	NUMBER OF ANIMALS	AGE, AVERAGE, AND RANGE, DAYS	STAGE OF CYCLE WHEN KILLED	MITOSES PER SQ. MM. AVERAGE ± S. E.
1	7	103 (61–204)	Proestrus	0.35 ± 0.01
2	11	87 (53–106)	Early estrus	1.41 ± 0.40
3	19	85 (52–126)	Late estrus	6.55 ± 1.07
4	14	94 (66–114)	Metestrus	1.37 ± 0.28
5	14	87 (66–114)	Diestrus	0.49 ± 0.12
6c	8	126 (67–172)	Proestrus	1.09 ± 0.31
7c	5	121 (89–142)	Early estrus	1.35 ± 0.36
8c	7	85 (60–111)	Late estrus	27.50 ± 9.87
9c	11	132 (117–139)	Late estrus	12.60 ± 3.31
10c	11	150 (140–169)	Late estrus	9.10 ± 1.75
11c	11	206 (171–242)	Late estrus	4.25 ± 1.09
12c	7	135 (127–146)	Metestrus	4.8 ± 0.80
13c	6	113 (79–142)	Diestrus	1.76 ± 0.26

There was considerable variation in the mitotic counts of the glands of animals killed in late estrus. In those not receiving colchicine this ranged from 1.5 to 21.7 mitoses per square millimeter with five of the nineteen animals having unusually low counts. The variability can be explained in part by the variation in the time at which a maximum cell division is attained. Most of the high counts were from animals killed 25 to 30 hours after cornified cells were first found in the vaginal smears, but a few high counts were from animals killed 5 to 10 hours earlier. Since the maximum activity is probably maintained for a few hours only, it may very well have been missed in the animals with the low counts. Thus, although there is undoubtedly individual variation, the average mitotic count for animals in late estrus would be higher than 6.6 mitoses per square millimeter if all the animals had been killed at a time of maximum mitotic activity.

The variability in mitotic activity associated with the age of the animal is shown by grouping according to age the forty animals receiving colchicine and killed in late estrus (groups 8c–11c, table 1). For the four age groups the average mitoses per square millimeter was as follows:

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[2] Groups 6c–13c received colchicine 9 hours before death.

AVERAGE AGE, DAYS	MITOSES PER SQUARE MILLIMETER
85	27.5
132	12.6
150	9.1
206	4.25

In a few individual cases older animals showed more mitoses than younger ones, but the difference between the 85- and 206-day age groups is highly significant statistically.

There was some evidence of a diurnal mitotic rhythm in the thyroid. Of the twenty-two animals in groups 9c and 10c (table 1), all of which had received colchicine 9 hours before death, seven were killed at 11 P.M., seven at 8 A.M., and eight at 5 P.M. The average mitoses per square millimeter for these was 19.6, 8.5, and 9.2, respectively. Although the difference between those killed at 11 P.M. and 8 A.M. is apparently considerable it is barely significant statistically (i.e., it might have been due to chance in about once in twenty times). However, whether or not this may be considered as evidence of a diurnal rhythm the fact remains that the estrous cycle is effective in causing an increased activity regardless of the time of day. It is interesting to note that in the hypophysis from these same animals the highest mitotic activity also occurred in animals killed at 11 P.M. (49 mitoses per sq. mm.) and the lowest in those killed at 8 A.M. (12.9 mitoses per sq. mm.).

Chouke and Blumenthal ('42) found there are generally more mitoses in the left lobe of the guinea pig thyroid than in the right lobe. This does not seem to be true for the rat. In thirty-one glands, selected because more numerous mitoses were present and the orientation was known, there were seventeen in which one side had more than 55% of the mitoses (five had more than 60%). Of the seventeen, nine had more on the left and eight, more on the right. The average mitoses per square millimeter in all the right lobes was 13.2 and in the left 13. Such minor differences can be accounted for by the errors in counting and are insignificant statistically.

The position of the dividing cells may have some bearing on the problem of the origin of new follicles in the thyroid. The majority, or 65% of 1215 dividing cells recorded for this purpose touched the lumen of the follicle and presumably replaced follicular cells that died. Most of the others were separated from the lumen by at least one non-dividing cell and appeared to be the beginning of a bud of a preexisting follicle. Very few divisions were seen in what might have been inter-follicular cells, so that it seems unlikely that new follicles would arise from them.

DISCUSSION

Since the greatest mitotic activity of the thyroid and hypophysis occurs in both glands during late estrus, it might be assumed that a single factor is responsible for the activity. In the thyroid, however, the increase and decrease in number of mitoses occurs somewhat earlier than it does in the hypophysis. For example, an animal killed in early estrus may show increased mitotic activity in the thyroid but not in the hypophysis. This suggests that the mitosis stimulating factor is not necessarily the same for the two glands. Further support for such an opinion has been obtained from experiments now in progress in which injections of estrogens result in an increase in mitoses in the hypophysis but not in the thyroid. It thus seems probable that the rise in mitotic activity of the thyroid during the estrous cycle is not the direct result of changes in the estrogen level but rather an indirect effect. That the effect is indirect, working through the hypophysis, is suggested by the work of Blumenthal and Loeb ('42) who found that administration of an acid extract or implants of the hypophysis resulted in an increased mitotic activity in the thyroid of the guinea pig.

During the estrous cycle in the guinea pig the time of highest mitotic activity in the thyroid (Chouke et al., '35, '42) occurs in what corresponds to the early part of diestrus in the rat. Since mitoses in the rat were at a low level in diestrus, it is difficult to find an explanation that accounts for the rise in both animals. The discrepancy, however, may be more apparent than real and be due to the difference in the timing of the phases of the estrous cycles in the two forms. There is also a possibility that the mitotic activity is an expression of physiological actions that occurred in the preceding cycle. Thus the factor that causes the increased activity might be the same but requires different lengths of time to become effective.

SUMMARY AND CONCLUSIONS

The mitotic activity of the thyroid gland of the female rat varies with the different phases of the estrous cycle. It is increased somewhat in early estrus, attains a maximum in late estrus, declines in metestrus, and is at a minimum in diestrus and proestrus. The maximum activity does not quite correspond in time with that in the hypophysis but is somewhat earlier. This and other evidence lead to the conclusion that the increased mitotic activity in the thyroid is not due to a direct effect of an ovarian hormone but rather to an indirect effect.

As the animals advance in age to 200 days there is a decline in mitotic activity so that the variation during the estrous cycle is hardly perceptible unless colchicine is given. There is some evidence of a diurnal rhythm, since more mitoses were found in colchicine injected animals killed at 11 P.M. than in such animals killed at 8 A.M. or 5 P.M.

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PERICHONDRIAL OSSIFICATION AND THE FATE OF THE PERICHONDRIUM WITH SPECIAL REFERENCE TO THAT OF THE OTIC CAPSULE

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FOUR FIGURES

Perichondrial ossification for most so-called cartilage or long bones is well described in most text books of histology. Long cartilage bones are foreshadowed by a growing shaft-like model of cartilage. Such a cartilage model first appears in the mid region of the future bone. It increases in length gradually by multiplication of its cells toward the ends of the shaft. This process of cartilage cell multiplication continues until the individual has attained full growth. The shaft like model of cartilage in the young fetus is surrounded by a layer of compact mesenchyma (shown at left of fig. 3) which is known as the perichondrium. These cells in this perichondium which lie next to the cartilage may themselves become cartilage cells and thus slightly increase the diameter of the model in its early stages of development. This augmentation in diameter is short lived as the center of the early cartilaginous model becomes quickly transformed into the diaphyseal ossification center with its enlarged lacuna and calcified matrix. This diaphyseal ossification center occurs in the early stages of the development of the cartilaginous model. Thus, new cartilage is forming at the ends of the model while the center is ossifying.

In response to this change in the cartilage model, the adjacent mesenchymal cells of the perichondrium metamorphose into osteoblasts which multiply rapidly and deposit osteoid tissue on the surface of the changed cartilage. This osteoid tissue quickly becomes calcified. These steps can be seen by reading figure 3 from left to right. With this transformation, the surrounding compact mesenchyme or developing fibrous tissue no longer surrounds cartilage but bone and is thus known as periosteum. Because of this, the generalized statement that the perichondrium becomes the periosteum as soon as the first perichondrial bone is formed has good morphological support and appears to be true for most bones. It is well to bear in mind that the transition from perichondrium to periosteum occurs at the time when it is still in a mesenchymal state and has not reached the stage of a specialized fibrous tissue.

The cartilaginous otic capsule and its perichondrium

The petrous portion of the temporal bone with its contained otic capsule is one of the cartilage bones. It differs from other bones in that it is neither a long bone nor a flat bone but rather a capsular or box-like bone enclosing the internal ear. The model or forerunner of this bone is the cartilaginous otic capsule. It makes its appearance in human fetuses of about 25 mm., crown rump length or at the age of 8 weeks. As the internal ear enlarges this capsule expands correspondingly. It remains cartilaginous until the internal ear has about attained its maximum size or at about the fourth or fifth month, after which the cartilage stops growing except at one or two minor spots and these are irrelevant to the question under consideration. The cartilage of the capsule therefore has attained its maximum growth before ossification begins.

The perichondrium during this period of growth and before ossification has set in, has begun to develop into a specialized capsular type of fibrous tissue. The innermost layer or two of cells next to the cartilage are somewhat rounded and resemble young cartilage cells. Outside of these the cells of the perichondrium become spread apart by the deposit of a collagenous fibrous matrix. The outermost part of the perichondrium retains more of its mesenchymal character. This is especially shown in the center picture of figure 4.

The advent of ossification and the fate of the perichondrium

Ossification centers appear in the cartilaginous otic capsule at various places. As many as fourteen such centers have been described. The ossification center, like those in other cartilage models, are characterized

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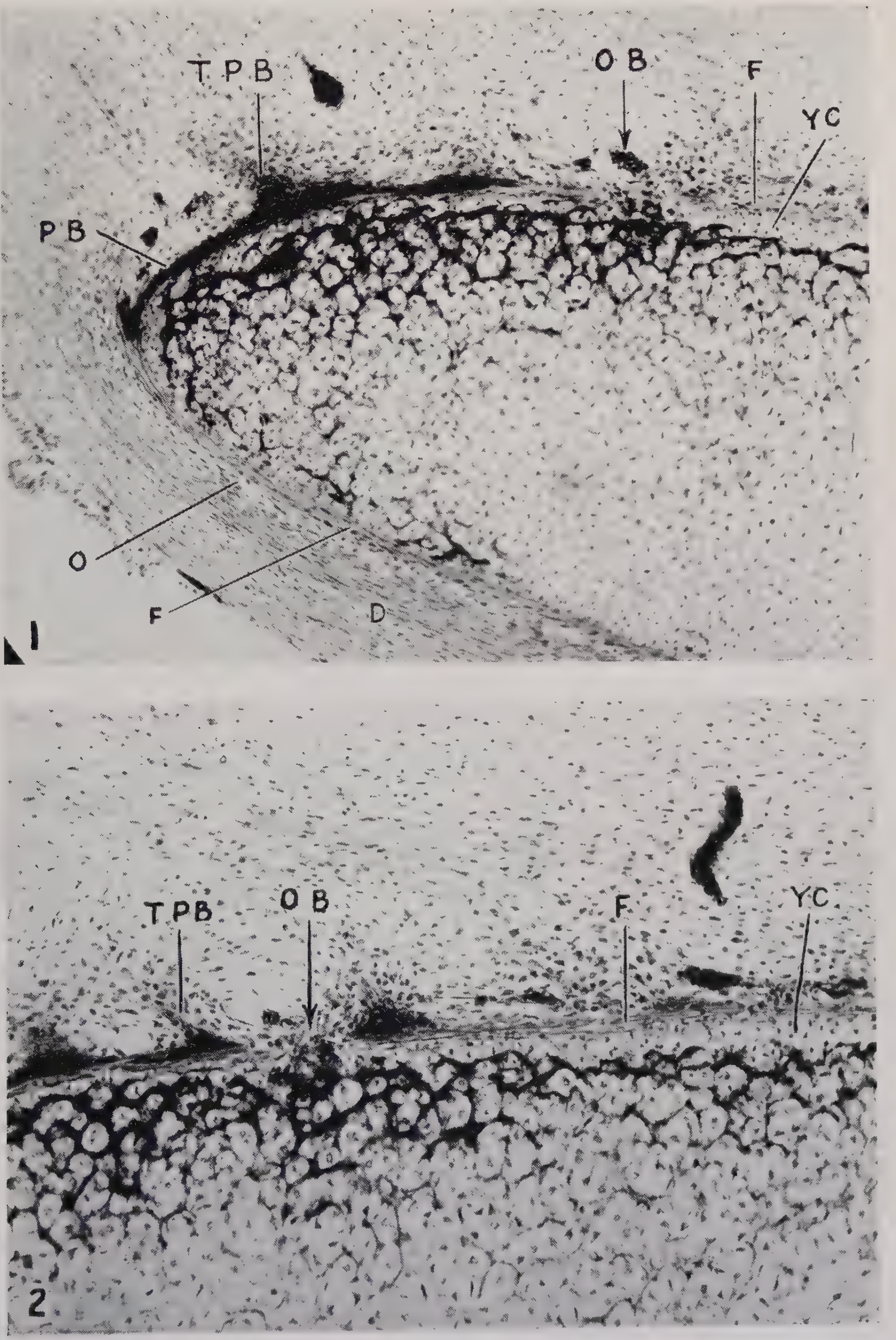
Fig. 1 Photomicrograph of a portion of the ossifying human otic capsule at the margin of the internal auditory meatus. (E. 38L — 147 mm. C.R. — slide 23 — section 2 — Bast collection.)

Fig. 2 Photomicrograph of an area similar to that shown in the upper part of figure 1. (E. 38L — 147 mm. C.R. — slide 22 — section 3. — Bast collection.)

Y.C., Young cartilage cell layer
F., Fibrous layer of perichondrium
O., Osteogenic layer of perichondrium
D., Dura
O.B., Osteogenic bud penetrating from the osteogenic layer through the ossifying fibrous layer into the calcifying cartilage
P.B., The fibrous layer transformed into perichondrial bone
T.P.B., Trabeculae of perichondrial bone forming in the osteogenic layer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Figures 1 and 2

by the enlargement of the lacunae and calcification of the matrix. The further transformation of the cartilage model has been previously described (Bast, '30). Our attention here is directed to the formation of the perichondrial bone and the part played by the perichondrium.

The perichondrium overlying an ossification center exhibits three or sometimes four distinct layers.

1. The young cartilage cell layer. This layer may be one to three cells in thickness. This layer, consisting of young cartilage cells with some collagenous fibers between them, can be distinctly seen lying between the calcified cartilage matrix of the ossification center and the rest of the perichondrium. This layer quickly changes into mature cartilage and its matrix becomes calcified and it thus becomes part of the cartilaginous ossification center. At the right in figure 2, this layer is seen as young cartilage whereas at the left, its matrix is being calcified.

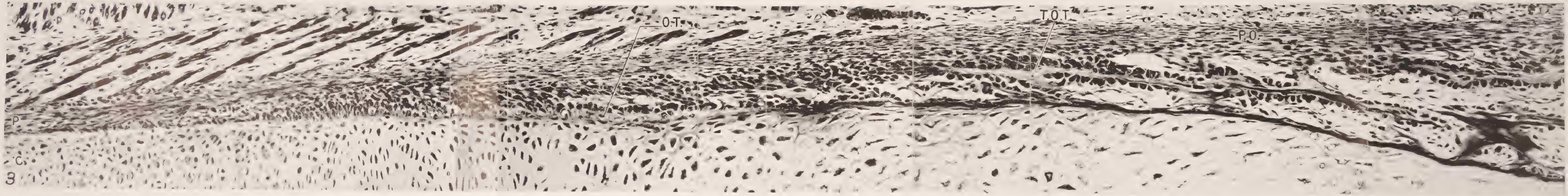
In the development of most other cartilage bone, it seems to be this layer which metamorphoses into an osteoblastic layer the cells of which multiply rapidly to form the osteogenic tissue that deposits perichondrial bone (fig. 3).

2. The dense collagenic layer. This layer of the perichondrium lies just outside the young cartilage cell layer. Its cells are spread apart and a dense matrix of collagenous fibers lies between them (figs. 1 and 2-F). This is the layer in which the first perichondrial bone is deposited. It is not clear whether some of the cells in this layer actually become osteoblasts and deposit bone about themselves or whether the calcareous deposit is dependent on the osteogenic layer just outside of this layer.

3. The mesenchymal layer. This gives rise to two sheets of tissue, (a) the osteogenic layer and (b) the periosteum.

(a) The osteogenic layer arises from the mesenchymal layer just outside the dense collagenic layer (figs. 1, 2, 4-O). Many capillaries invade this area and the cells are transformed into osteoblasts. The function of this layer is twofold; one, to give rise to osteogenic buds which will perforate the underlying layer and invade the modified cartilage or ossification center, and two, to proliferate osteogenic tissue in situ and thus to give rise to the perichondrial bone. In areas where perichondrial bone is being extensively formed, all of the mesenchymal layer may be transformed into this osteogenic layer (figs. 1 and 2, top).

(b) The periosteum as a rule develops from the outer part of the mesenchymal layer by the deposition of intercellular fibers. It overlies the osteogenic layer and the developing perichondrial bone (fig. 4). In



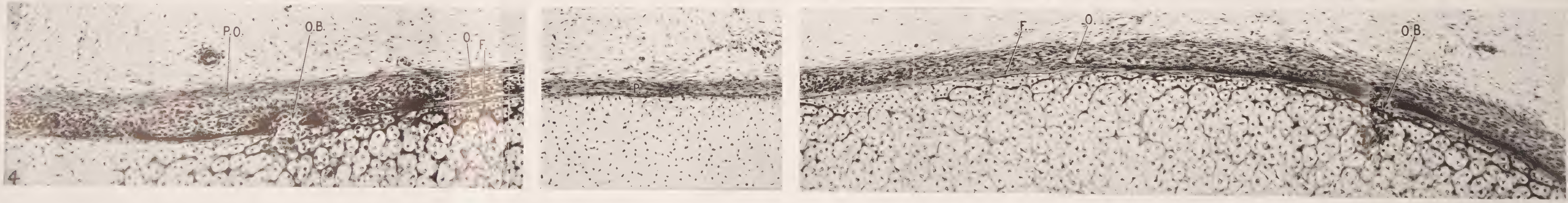


Fig. 3 A panoramic view of part of a rib of a woodchuck (Mossman collection) which shows the transformation of the primitive perichondrium into perichondrial bone and into periosteum. At the extreme left is shown the primitive perichondrium (P) consisting of compact mesenchymal cells overlying the rib cartilage (C). Reading the picture from left to right, one sees the transformation of the deeper layer of the perichondrium into osteogenic tissue and osteoblasts. Near the middle of the picture, at O.T., osteoid tissue is forming on the surface of the cartilage. Other trabeculae of osteoid tissue are seen forming in the osteogenic layer, at T.O.T. The dark staining osteoblasts are lined up on the surface of these trabeculae. Toward the right the osteoid tissue is changed into bone. The periosteum (P.O.) is seen to be continuous with the original perichondrium (P).

Fig. 4 This picture shows perichondrial ossification of the human petrous bone. (E. 36-L — 135 mm. C.R. — slide 10, section 2 — Bast collection.) The small center picture shows the perichondrium (P) of the cartilagenous otic capsule. It is comparable to the perichondrium of figure three except that next to the young cartilage cells the layer is fibrous and not cellular. Both to the right and left, the picture shows various stages in the ossification process and the zoning of the perichondrium and its transformation into perichondrial bone and periosteum. F. — Fibrous layer. O. — Osteogenic layer. P.O. — Periosteum. O.B. — Osteogenic bud penetrating from osteogenic layer through the ossifying fibrous layer into the calcifying cartilage.

places where the mesenchymal layer is all converted into osteogenic tissue, the periosteum later develops from the outer part of the osteogenic layer.

SUMMARY

In the development of most cartilage bones, the cells of the perichondrium which surround the cartilage model give rise to the osteogenic layer. The latter, in turn, forms the perichondrial bone. The outer, remaining portion of the perochondrium becomes the periosteum of the perichondrial bone.

In case of the petrous bone, the perichondrium of the otic capsule gives rise in its deeper part to a dense collagenic layer which forms the basic tissue in which the first perichondrial bone is deposited. Most of the rest of the perichondrium gives rise to the osteogenic tissue which is primarily responsible for the formation of the perichondrial bone. Only the outer part of the original perichondrium becomes the permanent periosteum.

THE EMBRYOLOGICAL DEVELOPMENT OF JACOBSON'S ORGAN IN DIDELPHYS VIRGINIANA (KERR)

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TWO FIGURES

The observations reported in this paper were made as part of a program to complete information concerning Didelphys virginiana, undertaken by the Zoology Department of the University of Pennsylvania and The Wistar Institute of Anatomy and Biology. The author wishes to express her gratitude to The Wistar Institute for permission to study its sets of slides, also to Dr. John McClain of Villanova College, and to Dr. Olin E. Nelsen for specimens of various ages for dissection.

The vomeronasal organ of Jacobson lies on either side of the nasal septum, supported by curved plates of cartilage. This structure was described and figured as early as 1703 by Rüysch; however, it bears the name of Jacobson who described it in mammals in 1811.

Many descriptions of Jacobson's organ in the marsupials have been written from the standpoint of comparative antomy. There are a few brief notes on its embryogeny in the opossum, mentioning only "that it is formed by the invagination of the epithelium into the septum" (McCrady).

The purpose of this brief paper is to describe the embryogeny and later development of Jacobson's organ in Didelphys virginiana.

The numbered stages mentioned below correspond to those used by McCrady in "The Embryology of the Opossum."

Stage 30 — is distinguished by the appearance of the oro-nasal groove.
Stage 31 — The oro-nasal groove is converted into a tube. The frontal process has developed. Median and lateral nasal processes have grown forward and are fusing with the maxillary process to form the beginning of the snout. (McCrady.)
Stage 32 — One can distinguish the cartilage of the nasal septum. A shallow indentation of the epithelial lining of the nasal cavity, on the antero-lateral surface of the nasal septum marks the anlage of Jacobson's organ.

Stage 33 — 34. The invagination becomes deeper and forms a diverticulum in the septum. Attached to the blind end of the sac one finds solid strands of tissue which stain deeply. Jacobson's cartilage, or the cartilage of the vomero-nasal organ appears at this time as a bar anterior to the invaginating tissue. A small blood vessel lying parallel to the nasal septum becomes associated with Jacobson's organ.

Stage 35 — The diverticulum elongates. The lumen of its anterior portion enlarges and becomes crescentic, while its opening towards the nasal passage narrows. The epithelial lining of the mesial wall of the sac begins to differentiate into columnar epithelium.

Birth occurs at the end of stage 35. Pouch young series have not been given stage names but are usually designated by crown-rump measurements. These were found to vary greatly from litter to litter, hence actual ages of the young will be indicated here.

One and one-half-day pouch young. Jacobson's organ is still a simple diverticulum. The epithelium of the mesial wall of the sac becomes high columnar epithelium. The cells along the lateral walls remain low, cuboid epithelium.

Jacobson's cartilage has become better developed, with the bars curving inward towards the nasal cavity. Eventually these curved bars completely encircle the anterior position of Jacobson's organ, forming a cartilaginous cylinder named Jacobson's capsule.

Two-day-old pouch young. No difference from previous stage.

Seven-day pouch young. At the cephalic end of the diverticulum the deeply staining solid strands of tissue seen in stage 34 have hollowed out to form one or two simple tubes lined with low epithelium. The opening of Jacobson's organ into the nasal cavity becomes narrowed by the development of the palatine processes. Eventually its opening comes to lie at the nasal end of Stenson's duct.

Seven to 15-day-old pouch young. These stages show the maximum development of Jacobson's organ. Its lumen becomes much enlarged and bean-shaped. At its cephalic end it communicates with several tubes whose development was marked in the previous stage. These simple tubes have become much elongated and branched at the cephalic end, forming a group of glands known as Jacobson's glands. Branches of the olfactory nerve pass through the cribriform plate and end either in Jacobson's glands or in the high columnar epithelium of the mesial surface of the organ. The expansion of Jacobson's organ, together with the flaring of the supporting cartilaginous bars, produce a marked protuberance on the anterior portion of the nasal septum (figs. 1 and 2).

As the palatine processes fuse under the nasal septum, the openings of Jacobson's organ on each side become further narrowed, or even totally closed. In some groups of animals Jacobson's organ opens directly into the nasal or oral cavity. In Didelphys, however, Jacobson's organ opens into the cephalic end of Stenson's duct which forms the upper part of the incisive canal on each side, opening into the oral cavity in the vicinity of the median papilla.

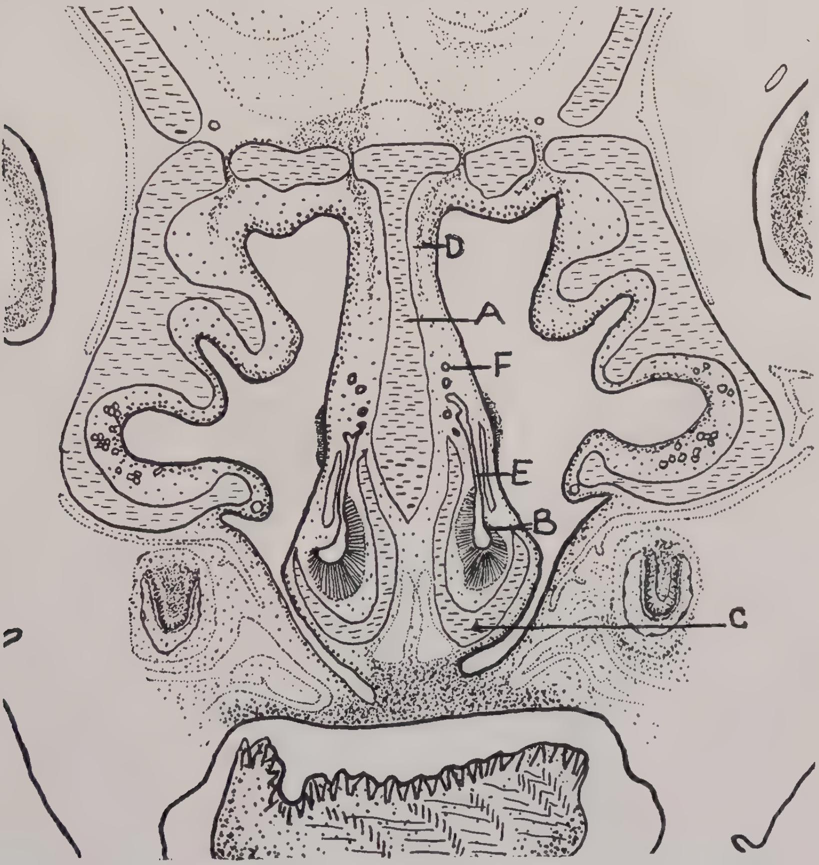

Fig. 1 Fifteen-day-old pouch young.

(A) Cartilage of nasal septum.
(B) Jacobson's organ.
(C) Cartilage of Jacobson's organ.
(D) Branch of the olfactory nerve.
(E) Duct leading to numerous glands.
(F) Glands of Jacobson's organ.

A 109-day-old opossum. In this specimen Jacobson's organ is a large flat tube supplied at its cephalic end with numerous glands. It lies in a cylinder of cartilage lateral to the nasal septum on the floor of the nasal cavity. Its opening into Stenson's duct may be completely closed.

Much of the voluminous literature on Jacobson's organ is concerned with its evolutionary significance, or with the probable use of a structure which is accessory to the nose. One of the most striking things about this organ is its essential similarity in all the groups in which it appears. "In all, Jacobson's organ is alike — a tube lined with sensory epithelium lying in the rostral oral end of the septum nasi. Because it is associated constantly with the vomer bone it has been called the vomero-nasal organ. It is associated with glands (Jacobson's glands) and is fed from above by branches of the olfactory nerve which enter the sensory epithelium. It opens either into Stenson's duct or into the floor of the nasal cavity, either above, or below, but always near Stenson's duct." (Mihalkovics.)

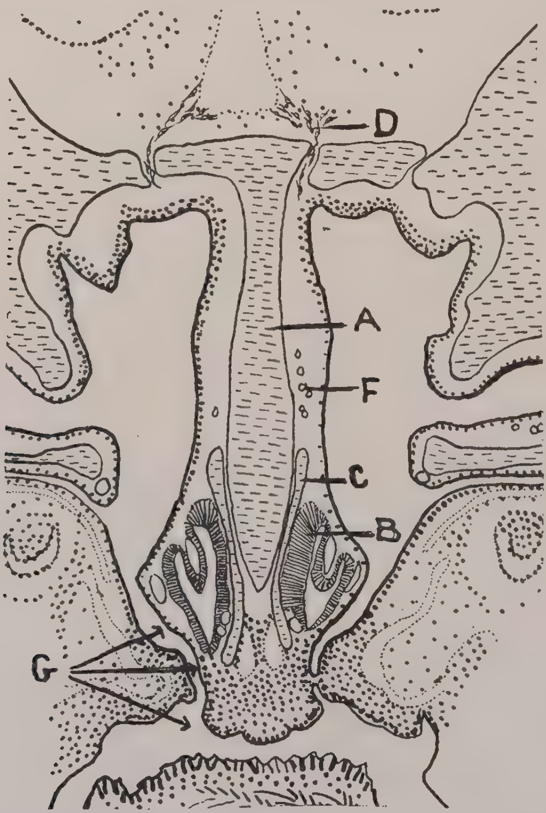

Fig. 2 Fifteen-day-old pouch young. Labels as in figure 1.
(G) Stenson's duct, or the naso-palatine canal.

Note in these figures the protuberance on the wall of the nasal septum caused by the enlargement of Jacobson's organ, and by the flaring of Jacobson's cartilage.

SUMMARY

1. Jacobson's organ arises as an invagination of epithelium into the nasal septum in stage 32.
2. The diverticulum thus formed continues cephalad, becomes associated with a series of tubes and glands. Cartilaginous bars surround it anteriorly. Branches of the olfactory nerve end in it.
3. The highest development of this organ is reached in 15-day-old pouch young.
4. In later stages it comes to lie in the nasal septum connected anteriorly to Stenson's duct which opens by way of the incisive canal into the mouth cavity.

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STRATIFICATION OF THE RAT ERYTHROCYTE BY ULTRACENTRIFUGING [1]

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ONE PLATE (THREE FIGURES)

Few cells have been so extensively studied as the mammalian erythrocyte. Notwithstanding all these studies, however, its structure remains to date a subject of confusion and decided controversy. This is due in part to the difficulty of harmonizing the morphological observations on the architecture of the erythrocyte with the studies concerning its physiology. For example, the shape of the erythrocyte alone presents a problem that is difficult to understand; some investigators hold that its shape is determined by the stroma while others attribute it to the surface membrane. Ponder ('24), after reviewing the literature on this subject, has summed up the present position concerning the structure of the erythrocyte when he said "It is obvious that the question cannot be decided until more accurate information is available."

Because of the need for further information concerning the structure of the erythrocyte, we are presenting this note describing the stratification that occurs following ultracentrifugation.

MATERIAL AND METHODS

The spleen of adult white rats was centrifuged at approximately 400,000 times gravity for 30 minutes. It was then fixed immediately in Champy's or in Bouin's solution. Sections were later stained in Heidenhain's hematoxylin and fast green, eosin B, or in Mallory's triple stain. Studies of ultracentrifuged spleen by supravital methods were also made.

OBSERVATIONS

In general, most of the erythrocytes are displaced to the centrifugal side of the spleen. Because of this it is easy to orient their position in relation to the centrifugal field of force. It should be mentioned, however, that the undisplaced erythrocytes in the sinusoids may show all of the characteristics herein described.

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[1] Aided by a grant from the Rockefeller Foundation for work in cellular physiology.

Figure 1 shows a section of ultracentrifuged spleen that has been stained in Heidenhain's hematoxylin. The centrifugal ends of the erythrocytes stain sharply, leaving the remaining part of the cell unstained. If such preparations are counterstained with fast green, the centripetal ends of the cells stain light green. Eosin B alone stains the centrifugal ends of the cells red. For the most part the cells appear stretched and seem to have lost their biconcave appearance. If this be true, it is logical to assume that the centrifugal force caused a breakdown of the internal structure when the shifting of the materials occurred. Furthermore, this technique seems to demonstrate two distinct substances in the erythrocyte which differ not only in their relative specific gravity but also in their staining reactions. Even more striking results are observed following the use of Mallory's triple stain. Here the centrifugal ends stain red and the centripetal ends, blue (fig. 2). Frequently, three layers are discernible following this method; namely, an orange layer at the centrifugal end, a red layer near the middle and a blue layer at the centripetal end (fig. 3). It is conceivable that a higher centrifugal force would produce a greater percentage of the cells showing three distinct layers. The centripetal ends of some of the erythrocytes appear irregular (fig. 3). Whether or not the membrane has been broken at this point or whether it represents an uneven contraction after removal from the centrifuge, it is difficult to determine.

Observations on supravital preparations of ultracentrifuged erythrocytes in the spleen show some evidence of stratification although it is not nearly so sharp as in the fixed and stained material. The hemoglobin in some of the cells appears to be shifted in the direction of the centrifugal field of force. The failure to reveal the various stratified layers in supravital preparations may possibly be due to their similar refractive index. Since nearly all the erythrocytes show stratification in fixed and stained preparations, it is unlikely, it seems to us, that the stratified substances represent such materials as the basophilic stippling or punctate basophilia. Furthermore, the stratified layers do not appear granular which probably means that the size of the particles making up the layers are of ultramicroscopic dimensions. This is supported by the fact that after dark field illumination (Salèn, '20) and ultraviolet light (Grawitz and Gruenberg, '06) studies on the erythrocyte, only a homogeneous, structureless protoplasm was revealed.

Fragmentation of the erythrocytes was also observed. Some were found which appear to have been pulled into two nearly equal parts, probably much in the same manner as occurs in Arbacia eggs (Harvey, '36). The great force required to stretch and pull the corpuscles into two parts indicates that they possess a relatively high viscosity and an elastic but not easily broken membrane (see also Seifriz, '27).

DISCUSSION

No effort will be made here to review the extensive literature on the red blood corpuscle. Excellent reviews dealing with the structure, physiology and chemistry of the erythrocyte have recently been published (Schäfer, '05; Cuff, '15; Ponder, '24; Gough, '24; Wintrobe, '30; Krumbhaar, '32; Isaacs, '38) and it is to these articles the reader is referred for references.

The observations herein reported for the erythrocyte can be repeated at will with essentially the same results. The question then seems to be, how far can we depend upon fixed and stained material for such studies? Generally speaking, both Champy's and Bouin's solutions are considered good cytological fixatives because they produce the minimum amount of artifact. Furthermore, we have found (Beams and King, '36) that other cells are not killed and readily recover after being centrifuged at forces 1,000,000 times gravity for 30 minutes. In addition, we have not observed a fibrous stroma in the erythrocytes which has been argued to be present as an artifact due to the coagulation of the proteins. However, in spite of the evidence supporting the conclusions drawn in this paper, we fully realize the desirability of subjecting centrifuged erythrocytes to various ultramicroscopic techniques which were not available to us.

Our observations on the erythrocytes do not support the view that is often expressed that the erythrocyte is a homogeneous, structureless mass of protoplasm. We, however, are at present unable to identify the various layers stratified. Test for lipoids by staining techniques have been of little value. Eosin has been used by some to stain hemoglobin in the erythrocyte but it is obviously not specific for it. It will be recalled that eosin stained the centrifugal end of stratified cells in the same part of the cell where the hemoglobin is observed in centrifuged supravital preparations.

SUMMARY

Ultracentrifuged rat spleen shows the erythrocytes to be sharply stratified into two or three layers that can be selectively stained by Heidenhain's hematoxylin and light green or by Mallory's triple stain. Such cells appear to be elongated in the direction of the centrifugal force and to have lost their biconcave appearance probably because of a breakdown of their intracellular architecture. Supravital preparations

fail to show the sharp stratification observed in the fixed and stained preparations although some of the cells do show a displacement of the hemoglobin. These results do not support the view that the erythrocyte of the rat is composed of a structureless, homogeneous mass of protoplasm, but indicate that they are composed of at least three discrete substances which differ in their relative specific gravity as well as in their staining reactions after stratification.

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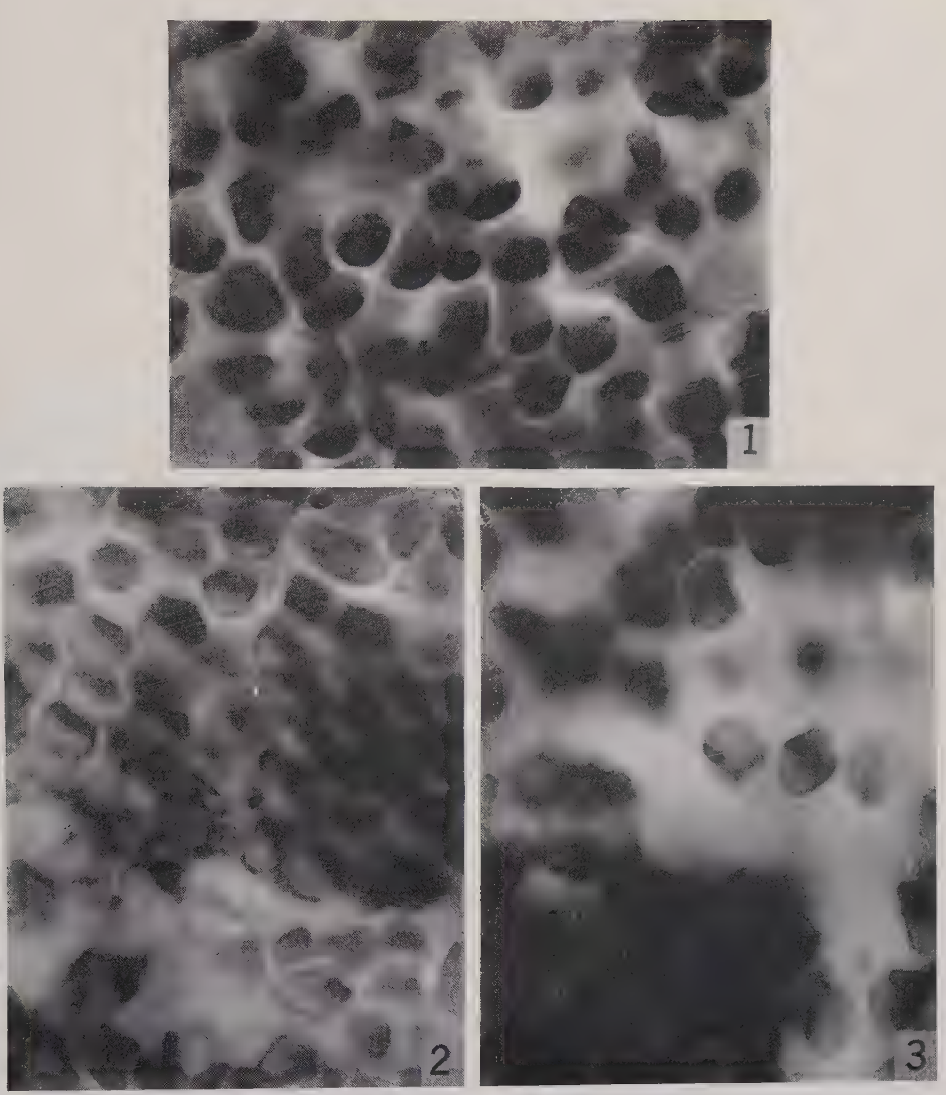

All figures are of centrifuged spleen showing the stratification of the erythrocytes. The direction of the centrifugal force is toward the bottom of the plate.

1 Heidenhain's hematoxylin stained erythrocytes showing a dark layer at the centrifugal ends.
2 Similar section stained in Mallory's triple stain. Here two distinct strata are visible.
3 Mallory's stained section with cell to right of center showing three distinct layers.

DISTRIBUTION OF MITOSES IN THE CORNEAL EPITHELIUM OF THE RABBIT AND THE RAT

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TWO FIGURES

This report on the frequency of cell division in the epithelial layers of the cornea of rabbits and of rats represents part of a larger study dealing with the effects of ultraviolet radiation on the tissues of the mammalian eye (Carnegie Institution of Washington Year Book No. 42, '43, p. 139). It has been known for many years that exposure to heavy doses of ultraviolet radiation may cause destruction of cells of the corneal epithelium (for example, Birch-Hirschfeld, '09; Verhoeff and Bell, '16; Cortese, '30; Miescher and Wiesli, '32), but relatively little is known of the fundamental nature of the antecedent changes or the minimum dosage necessary for their production. Such information is important, not only for an understanding of the effects of ultraviolet radiation on the living cell, but also for guidance in determining safe levels of exposure of the human eye to such radiation as emanates from the welder's arc (Rieke, '43) or the low-pressure mercury vapor lamps used for disinfection of air (Council on Physical Therapy, '43; Hollaender, '43).

One aspect of the problem, which can be approached by means of cytological techniques, is the measurement of disturbances produced by ultraviolet radiation in the normal cycle of mitosis. Efforts were made, therefore, to measure the effects of radiation of a given wave length on cell division in the epithelium of the cornea of one of the eyes of an experimental animal, using the other eye as a control. This material presents some complications, inasmuch as the impinging radiation must pass through the three or four superficial layers of the epithelium to reach the mitotically active basal layer. In the first experiments, intense treatment was given to insure penetration to this layer; for example, rabbit corneas were exposed to radiation of wave length 2537A for 5 to 10 minutes at an intensity of about 3000 ergs per square centimeter per second. Such treatment disrupts the normal cycle of mitosis, so that in corneas fixed 6 to 8 hours after radiation no metaphases or anaphases were found; instead there were numerous stages resembling prophases, and an approximately equal number of cells with more or less indiscernible chromosomes but highly refractile nucleolus-like bodies. A similar experiment in which the cornea of a Rhesus monkey (Macacus mulatta) was used showed a much higher proportion of abnormal mitoses (about 5% prophases to 95% abnormals). In the treated rabbit corneas these aberrant mitoses alone were as frequent as the total number of dividing cells in the control corneas, and in the monkey they were twice as frequent as in the control. From this limited evidence it appeared that the ultraviolet radiation had an effect in blocking mitosis, presumably in the prophase, but that some cells in earlier prophase stages or in the interphase had continued the mitotic process until later prophase stages were reached. For a detailed analysis of these specific effects, especially following doses of low intensity, it was evident that more favorable material was required, at least in the preliminary experiments; and it was decided to make use of the neuroblasts of the grasshopper, Chortophaga, which Carlson ('38) had found suitable for similar studies with x-rays. The results of experiments using this material are reported elsewhere (Carnegie Institution of Washington Year Book No. 42, '43, p. 140; Carlson and Hollaender, '44).

In the work outlined above with the rabbit and the monkey, and in other preliminary experiments with the eyes of the mouse, rat, and cat, we had observed that there was considerable variability in the distribution of dividing cells in the epithelium of a single cornea, and between the two corneas of a single animal. The latter finding was not in accord with the statements of Kornfeld ('22), Politzer ('25), and Gurwitsch and Aniken ('28) that the numbers of mitoses do not generally differ greatly in the two eyes of an animal, a conclusion based primarily on the study of amphibian material. Inasmuch as determination of the range of variability was essential to any further experiments involving comparisons of treated and control areas, and since we had developed a method facilitating inspection of the corneal epithelium, it was decided to make more extensive studies with New Zealand White rabbits and Albino rats, both of which were obtainable in inbred stocks that could be maintained for further experimental work. The results of these studies are presented in the following paragraphs.

METHODS

The cells of the corneal epithelium are arranged in four or five layers that rest on the underlying connective tissue. Under normal conditions mitoses are found in the basal epithelial layers. To facilitate identification and counting of the various stages of division, flat preparations of entire corneas were used. Based on the pioneer work of Kornfeld ('22), Politzer ('25), and Gurwitsch and Aniken ('28) with corneas of salamander larvae, we have developed a rapid method that involves staining in acetic orcein of the entire mammalian cornea, or of its epithelium after separation from the underlying tissues, flattening, and mounting in a euparal-like medium. The advantages of these flat preparations lie in the rapidity with which material may be secured for microscopical observation, the freedom from the exacting routine of sectioning the refractory material, and the accuracy that is possible in comparing rates of mitosis in different areas.[1]

In order to minimize such differences in the frequency of mitosis as might be attributable to decreased food intake (as Kornfeld, '22, found in salamanders) or to a diurnal rhythm (as Picón, '33, and Carleton, '34, have reported for the mouse), mature, well-fed animals were used in these studies and were killed during the mid-day period. Most of the animals were decapitated; the eyes were removed promptly and transferred to the fixative. Since at the most a very few minutes elapsed from the time the animal was first handled until the eyes reached the fixing fluid, there was little opportunity for emotional or physiological disturbances to influence the rate of mitosis.

Various of the standard cytological fixatives, such as Zenker's, Carnoy's, and Navashin's fluids, were tried but subsequently discarded in favor of an absolute alcohol, glacial acetic acid mixture, in the proportions of about 3.5 to 1 (McClintock, '29). After the eyes were placed in this fluid a few minutes were allowed to permit the surface layers of the epithelium to harden before the cornea was severed from the posterior portion of the eye by cutting circumferentially close to the sclerocorneal junction. Satisfactory fixation of the epithelium was obtained within a few hours, but if desired the material may be stored in the refrigerator for indefinite periods of time until needed.

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[1] The use of flat preparations of strips of the cornea of the rat has been reported recently by Buschke, Friedenwald, and Fleischmann ('43), who, following the example of Gurwitsch and Aniken, stained this material with a hematoxylin dye. In the early stages of our work we tried hematoxylin and a series of aniline dyes, but rejected them in favor of orcein, since the techniques were more laborious, and the stains seemed less selective in their coloration of the chromatic materials of the cell.

The cornea was subsequently freed of any adherent conjunctiva, iris, or ciliary body. Since it was desired to maintain the cornea as a unit, four or five incisions equidistant along the circumference and extending toward the center were made at this time in order to permit subsequent flattening without undue buckling of the epithelial layers. The material was then transferred to a solution of orcein in acetic acid (LaCour, '41) where it remained about 20 minutes. Duration of the staining period must be judged according to the quality and concentration of the dye. We have been using a natural (not synthetic) product, which had proved very satisfactory for fixing and staining the salivary-gland chromosomes of Drosophila. To each 100 ml. of 60% acetic acid, 2 gm. of orcein were added; the solution was boiled for an hour or more with the aid of a reflux condenser and then filtered. If entire corneas are kept flat during the staining period, less difficulty will be encountered in subsequent handling. Flattening may be induced by placing the material on a slide, in a few drops of stain, under a weighted cover slip. Fluids can be drawn off at one side of the cover and replaced at the other in the process of dehydration.

The excess stain was rinsed off with 45% acetic acid, and the material then passed through 70 and 95% alcohols for further destaining and for dehydration. The gum sandarac mounting medium which we have used is miscible with 95% alcohol, and has been described by Buchholz ('38). Following these procedures, the chromatin of dividing cells should be colored, all other cell components remaining uncolored and relatively transparent. If desired, a 0.5% solution of orange G in 95% alcohol may be used as a counterstain.

Preparations obtained by the methods described are quite thick: about 180 microns in the rat, and about 280 in the rabbit, of which the epithelium alone comprises only 40 to 50 microns. Beneath the four or five layers of epithelial cells is the extensive zone of connective tissue comprising the substantia propria. Naturally transparent in the normal animal, this tissue, despite its thickness, does not interfere seriously with observations made by means of high-powered dry objectives, although intense illumination is required. Moreover, since the orcein colors only chromatin materials (resembling the Feulgen reaction in this respect), the mitoses in the underlying epithelial cells are not obscured by intense color in the cytoplasm of cells of the more superficial layers (fig. 2).

When thinner preparations were desired, especially of rabbit corneas, the major portion of the substantia propria was removed before the marginal serrations were made. The cornea was transferred to 30%

alcohol, and the epithelium and the adjoining connective tissue were then peeled from the bulk of the substantia propria by gradually working blunt-tipped forceps between its upper strata.

In preparing the slides for observation, the parts of the cornea to be surveyed were outlined by very thin lines placed on the cover glass with a ruling pen. Two patterns were used, depending on whether the major portion of the cornea was to be examined, or only a sample thereof. In the first case, three or four intersecting diameters were drawn, with a series of connecting lines, to furnish a spiderweb type of pattern (fig. 1). Each of the six or eight resulting wedge-shaped sectors was lettered A to F, or A to H, and the subdivisions numbered 1 to 4. By counting mitoses within the circumscribed regions it was possible to make comparisons between adjacent sectors and concentric rings, although in the latter case there was considerable inequality in size between the central and peripheral zones. In making sample measurements, all the mitoses were counted in a narrow meridional strip that was ruled into squares by connecting lines. A second strip laid off at right angles to the first permits comparison of equal areas along two different axes (fig. 1).

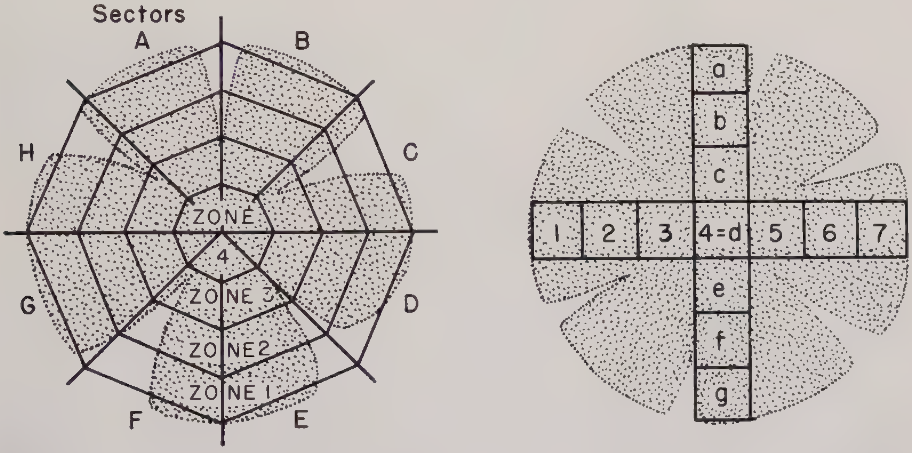

Fig. 1 Types of markings placed on cover glasses to facilitate counting of mitoses in the epithelium of different regions of the cornea.

The areas encompassed by the ruled lines were determined precisely by planimeter measurements of camera lucida drawings. Most of the observations were made by means of a 3-mm., 60X-objective, and 12.5X-oculars. To facilitate the counting process, three fine hairs, arranged in the form of an H, were cemented to the diaphragm of one of the oculars. The parallel hairs, placed horizontally, delimited the field;

the cross-hair served as a line of separation between counted and uncounted cells as they were moved across the field by manipulation of the mechanical stage. (An ocular net micrometer will serve the same purpose, although the large number of engraved lines is more confusing.) By careful focussing, it is a simple matter to restrict the counts to the areas outlined on the cover glass.

Observation of the purplish, orcein-colored chromosomes is facilitated by use of a green filter, such as a Wratten, no. 61, but green-colored cellulose acetate provides an inexpensive and fairly satisfactory substitute (Carlson, '38).

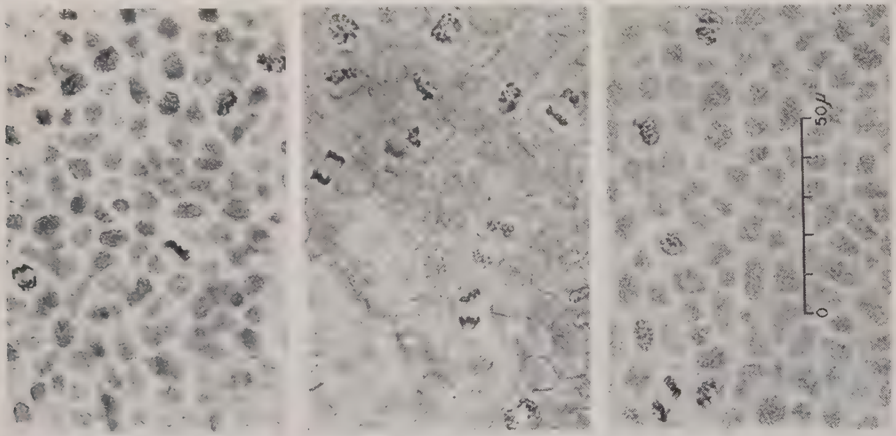

Fig. 2 Photographs indicating abundance of dividing cells found in the basal layer of the epithelium of the cornea of the Albino rat.

THE DISTRIBUTION OF DIVIDING CELLS

In the rabbit. Three contiguous regions of a single cornea, each about 20 sq. mm. in area, which were examined in a preliminary survey, gave counts of 37, 48 and 62 mitoses per square millimeter. It is evident that regional differences within a single cornea may be great, even when areas as large as one-seventh to one-eighth of the total are considered (the average surface area of the cornea of the New Zealand White rabbit is about 150 sq. mm.). In these preliminary surveys all stages of mitosis from early prophase to late telophase were scored, and from the counts so obtained it was apparent that, despite great variability in the number of dividing cells per unit area, the proportions of the different stages of mitosis are relatively constant from region to region (page 174). Reliable comparisons could accordingly be based on counts of the more conspicuous stages, such as metaphases and anaphases. Adopting this procedure, a series of meridional strips were surveyed; the counts obtained in two typical cases are presented in table 1. Most commonly the mitoses were least abundant in the peripheral regions and increased along a gradient toward the center. The examples presented in table 1 show, however, that there is not always a single gradient from periphery to center, and that the counts made along the two arms of a single strip are not always similar. Distribution of the dividing cells along these meridional strips is obviously not at random; application of the chi square test (χ^2) reveals that the departure from randomness cannot be attributed solely to the ever-present sampling error. The probability (P) is less than 0.000005 that the counts obtained depart solely because of random sampling from those to be expected on the hypothesis of uniform density of mitoses, whereas values of P higher than 0.05 are in general considered requisite for confirmation or rejection of the hypothesis tested.

TABLE 1

Distribution of mitoses in meridional strips of corneal epithelium of rabbits (adult New Zealand White ♀♀, each weighing ca. 3.0 kg.).

Zones in meridional strips

(NUMBER OF METAPHASES/AREA IN SQ. MM.)					TOTALS
44/2.55 17.3	98/6.12 16.0	234/6.12 38.2	232/6.29 36.9	44/1.87 23.5	652/22.95 28.4
102/5.16 19.8	357/11.86 30.1	296/12.10 24.5	347/12.61 27.5	81/5.05 16.0	1183/46.78 25.3

Differences in counts obtained along the two arms of a meridional strip suggested that analyses be made along horizontal and vertical axes that were determined with respect to the neutral position of the eye. Measurements made in this way on a pair of corneas are presented in table 2. In three of the four strips analyzed the counts obtained depart significantly from those expected on the hypothesis of uniform density ($P<0.01$ in these cases), but in the strip measured along the vertical axis of the right cornea there is no appreciable departure, the χ^2 test giving a value of P of about 0.99. When total counts in the two strips of each eye are compared with respect to the areas involved (a comparison of density of mitoses), the differences observed are no greater than may be attributed to the sampling error; comparing horizontal and vertical axes of each cornea by the χ^2 method gave a value of P of about 0.7 for the left cornea and between 0.5 and 0.3 for the right cornea. The difference between the two eyes of this animal, as revealed by the total counts along the four sample strips, is, however, significant ($P<0.01$).

More detailed analyses, with respect to small areas, were made on several additional corneas which had been marked off into sectors and zones (as diagrammed in fig. 1). Counts on four of these are presented in tables 3 and 4. The area surveyed in each cornea falls short of the total of about 150 sq. mm.; this is due in part to exclusion of the peripheral region adjoining the sclerocorneal junction, and in part to the masking of some cells by the lines drawn on the cover glass. Differences among sectors and zones are in each cornea greater than may be attributed to sampling errors, since χ^2 tests gave values of P much lower than 0.01. The density of mitoses from zone to zone differs markedly, with a sharp gradient rising toward the center. Differences among the small subdivisions are so great that only total counts for each sector and zone have been given in tables 3 and 4.

TABLE 2

Distribution of mitotic cells in meridional strips of corneal epithelium along predetermined axes of eyes of an adult ♀ New Zealand White rabbit, weight ca. 3.5 kg.

Zones in meridional strips

	(NUMBER OF METAPHASES/AREA IN SQ. MM.)					TOTALS
Left eye (Horizontal axis). Zone near medial canthus to left	107/6.94	191/7.33	169/5.07	121/4.39	93/5.81	681/29.54
	15.4	26.1	33.3	27.6	16.0	23.1
Left eye (Vertical axis). Upper region to left	50/2.88	88/3.01	115/4.21	75/3.95	61/3.19	389/17.24
	17.4	29.2	27.3	19.0	19.1	22.6
Right eye (Horizontal axis). Zone near medial canthus to left	75/4.59	60/4.67	105/4.79	93/4.50	83/4.59	416/23.14
	16.3	12.8	21.9	20.7	18.1	18.0
Right eye (Vertical axis). Upper region to left	80/4.37	84/4.34	85/4.29	80/4.12	95/4.91	424/22.03
	18.3	19.4	19.8	19.4	19.3	19.2

The two corneas of each of these animals also differ significantly; comparison of the "Totals" for left and right corneas of animal no. 9–21 (table 3) by the χ^2 method gave a value of $P<0.01$, and a similar comparison of the corneas of animal no. 8–13 gave P much less than 0.01. However, in order that comparisons of numbers of mitoses per unit area may have validity in measuring frequency of mitoses, it is

TABLE 3

Number of mitoses per square millimeter in different regions of the corneal epithelium of New Zealand White ♀ rabbit no. 9–21.

Sectors

	A	B	C	D	E	F	G	H
Left eye	143/7.5 19.0	183/7.5 24.3	300/14.0 21.4	167/6.5 25.8	256/9.8 26.0	291/12.1 24.0	69/3.9 17.6	178/8.0 22.1
Right eye	343/17.7 19.4	246/13.6 18.1	118/6.3 18.6	121/7.3 16.7	252/10.2 24.7	317/15.9 20.0	352/13.4 26.2	253/11.8 21.4

Zones

	1	2	3	4	TOTALS
Left eye	167/11.5 14.6	732/32.6 22.5	549/21.0 26.1	139/4.4 31.4	1587/69.5 22.8
Right eye	464/31.5 14.7	713/36.5 19.5	578/21.8 26.5	247/6.4 38.5	2002/96.2 20.8

TABLE 4

Number of mitoses per square millimeter in different regions of the corneal epithelium of New Zealand White ♀ rabbit no. 8–13.

Sectors

	A	B	C	D	E	F
Left cornea	312/18.7 18.6	155/8.0 19.4	444/18.6 23.9	353/17.5 20.1	269/11.9 22.6	328/13.1 25.1
Right cornea	586/18.5 31.7	223/7.4 30.3	583/20.0 29.1	455/18.4 24.7	400/16.8 23.9	502/19.0 26.4

Zones

	1	2	3	4	TOTALS
Left cornea	294/24.7 11.9	784/34.2 22.9	543/19.3 28.1	240/7.6 31.6	1861/85.8 21.7
Right cornea	765/35.5 21.6	1019/38.3 26.6	751/21.1 35.7	214/5.2 41.0	2749/100.0 27.5

essential that equal areas contain equal numbers of cells. Measurements were made, therefore, of the area covered by several hundred cells in the basal layer in various parts of these four corneas. Averages in square millimeters, for units of 1000 cells in the four zones of each cornea are:

Rabbit no. 9–21, left cornea 0.0332; 0.0340; 0.0350; 0.0342 (Average 0.0341)
right cornea 0.0326; 0.0330; 0.0312; 0.0313 (Average 0.0320)
Rabbit no. 8–13, left cornea 0.0291; 0.0294; 0.0273; 0.0273 (Average 0.0283)
right cornea 0.0362; 0.0347; 0.0351; 0.0353 (Average 0.0353)

Marked differences in the size of cells of the two corneas of an animal, as in rabbit no. 8–13, are probably attributable to differential effects of fixation and dehydration. Taking cell size into consideration, the following frequencies were calculated:

no. 9–21, left — 1 mitosis for each 1284 cells of the basal epithelial layer
right — 1 mitosis for each 1501 cells of the basal epithelial layer
no. 8–13, left — 1 mitosis for each 1630 cells of the basal epithelial layer
right — 1 mitosis for each 1031 cells of the basal epithelial layer

In rabbit no. 9–21 the ratio of frequency of mitoses (1 in 1284 as compared with 1 in 1501) is 1.08 to 0.92, as contrasted with the ratio of 1.046 to 0.954 obtained when density of mitoses in these two corneas is compared (22.8 mitoses per square millimeter and 20.8 per square millimeter). Likewise in rabbit no. 8–13, the frequencies for left and right corneas stand in a ratio of 0.77 to 1.23 (1 in 1630 compared with 1 in 1031), whereas the ratio for density of mitoses (21.7 mitoses per square millimeter compared with 27.5 per square millimeter) is 0.88 to 1.12. It is thus apparent that the frequency of cell division in the corneal epithelium of the two eyes of an animal may vary more widely than is apparent from calculations of density of mitoses alone.

Distribution in the Albino rat. A series of measurements, similar to those outlined above, were made on corneas of Albino rats. Their smaller size as compared with rabbit corneas (the surface area is about 35 sq. mm.), and higher percentage of dividing cells, facilitated these observations.

The results of counts of four corneas are given in summarized form in tables 5 and 6. It is apparent from casual inspection of these figures that there is more uniformity in the distribution of dividing cells throughout the cornea than was observed in the rabbit. The distribution reported in the lower half of table 5, along two meridional strips of a cornea, is essentially at random, the differences in number from region to region being no greater than might be attributed to the sampling error (the value of P, obtained from χ^2, is between 0.91 and 0.74 for the vertical axis, and between 0.56 and 0.41 for the horizontal axis). However, mitoses are not distributed at random along the vertical axis of the cornea of the other eye shown in this table (P between 0.0005 and 0.0003), and distribution along the horizontal axis is just at the threshold of significance (P about 0.05). Total counts made along either the horizontal or the vertical axis of each of these corneas may be regarded as furnishing a representative sample, since differences between them fall within the range of the sampling error. Comparison of the two axes of one eye (lower half of table 5) by the χ^2 method gave a value of P between 0.20 and 0.10, and comparison of the two axes of the other eye gave a value for P between 0.05 and 0.02.

A more detailed analysis of the density of mitoses within the right and left corneas of a single rat is presented in table 6. The χ^2 test again shows that the distribution departs significantly from randomness when sectors A to H are compared ($P<0.000001$ for the left cornea, and <0.000095 for the right cornea), and when zones 2, 3 and 4 are compared ($P<0.002$ for the left cornea and <0.000553 for the right cornea). Within each cornea the mitoses are more abundant in the

TABLE 5

Distribution of metaphase stages of mitosis in meridional strips of corneal epithelium of 2 adult ♀ Albino rats, weight of each ca. 400 gm.

Zones in meridional strips

	(NUMBER OF METAPHASES/AREA IN SQ. MM.)					TOTALS
Horizontal axis	107/0.85 125.9	92/0.72 127.8	103/0.82 125.6	86/0.74 116.2	72/0.85 84.7	460/3.98 115.6
Vertical axis	51/0.76 67.1	92/0.82 112.2	103/0.82 125.6	81/0.69 117.4	72/0.91 79.1	399/4.00 99.8
Horizontal axis	102/1.08 94.4	148/1.94 76.3	158/1.73 91.3	95/1.12 84.8	98/1.13 86.7	601/7.00 85.9
Vertical axis	104/1.00 104	117/1.26 92.9	93/0.97 95.9	89/0.97 91.8	79/0.90 87.8	482/5.10 94.5

TABLE 6

Number of mitoses per square millimeter in different regions of two corneas of an adult ♀ Albino rat.

Sectors

	A	B	C	D	E	F	G	H
Left cornea	924/2.6 362.4	659/1.9 341.5	406/1.3 324.8	903/2.1 423.9	372/1.3 295.2	933/2.7 344.3	777/2.0 388.5	877/2.4 368.5
Right cornea	490/1.2 401.6	704/1.6 448.4	821/1.6 503.7	799/1.6 490.2	608/1.4 437.4	849/2.1 404.3	499/1.2 433.9	721/1.8 447.5

Zones

	2	3	4	TOTALS
Left cornea	2948/8.5 346.0	2175/5.8 373.7	728/1.9 389.3	5851/16.2 361.0
Right cornea	3010/7.1 426.4	1906/4.1 467.2	646/1.3 489.4	5562/12.5 446.4

center than at the periphery, but the gradient is much less steep than in the rabbit.

The numbers of mitoses per unit area are significantly different in the two corneas of this rat (the χ^2 test yields P that is much less than 0.01), and stand in the ratio of 1.106 to 0.894. In order to compare frequencies of mitoses in the two corneas, measurements of cell size were made in each of the three zones, with the following results:

Left cornea, area in square millimeters per 1000 cells — 0.0422; 0.0426; 0.0429, or about 1 division figure for each 65 cells. Right cornea, area in square millimeters per 1000 cells — 0.0468; 0.0484; 0.0485, or about 1 division figure in each 47 cells. These frequencies are in the ratio of 1.16 to 0.84. As was found in the rabbit, it is again shown that the factor of cell size should be considered in critical comparisons of the number of mitoses in the corneal epithelium of the two eyes of an animal.

General considerations. Determination of the frequency of mitoses in the corneal epithelium by survey of meridional strips and whole corneas has given some indication of the range of variability that must be considered in experimental work involving comparison of numbers of dividing cells in the two eyes of an animal. The survey has also revealed that the Albino rat is a more favorable experimental animal than the New Zealand White rabbit for studies such as we have undertaken to measure the effects of radiation on the mitoses of the corneal epithelium.

The frequency of mitoses in the cornea of the rat was found to be higher than in any of the other animals investigated, including rabbits, mice, cats and a monkey. The overall frequency in the rat was about 19 to 20 times that in the rabbit, although the contrast is not so great when maximum numbers in small areas are compared. For example, we have found as many as 650 dividing cells per square millimeter in the rat, and 173 in the rabbit. Our counts on the rat, although higher than those of Buschke, Friedenwald and Fleischmann, which were also made on flat preparations, fall short of the maximum of 811 per square millimeter reported by Arey and Covode ('43) from their study of sectioned corneas. These numbers, however, are all of the same order of magnitude; and differences in counts from animal to animal are to be expected, not only on the basis of their genetic composition and the environmental influences to which they have been subjected, but also because of such subjective factors as the scoring technique, which will depend in part on the quality of the preparation employed.

Within a single cornea the frequency of mitoses may differ considerably from area to area. As few as 7 mitoses have been counted in 1 sq. mm. of a rabbit cornea, and as many as 55 in a similar area in another part of the same cornea, whereas in different parts of a rat cornea the maximum number of 625 was not twice the minimum of 376. Whatever may be the stimulus that promotes cell division in localized areas, it often continues through more than one mitotic cycle, so that groups of dividing cells occur, separated by regions devoid of mitoses. Such regions are more extensive in the rabbit cornea than in that of the rat, and counts within small areas are accordingly more variable.

In larger areas, especially the cornea as a whole, there is in general a gradient from the periphery toward the center, where the mitoses are most abundant. The gradient is slight in the rat, and more pronounced in the large cornea of the rabbit. This is the situation which obtains in the dog, according to the single count reported by Gurwitsch and Aniken ('28). In some of the corneas, particularly in the rat, mitoses were most abundant in the vicinity of the limbus; but we have not been able to confirm the statement of Buschke et al. that mitoses are regularly more numerous peripherally than in the center.

Inasmuch as the counting of mitoses in the entire cornea is both laborious and time-consuming, some sampling procedure that will give representative values is to be preferred for experimental work. Because of the gradient from periphery to center, concentric areas may differ markedly. Wedge-shaped sectors extending from the center to the periphery may also differ significantly. More uniformity was found, however, between meridional bands traversing the cornea, provided each encompassed about one-tenth of the total area. Narrower strips did not always give such uniform distribution. Since the various stages of mitosis in the untreated animal were found to be in almost constant proportions when large numbers of cells were considered, it appears that a more reliable index of frequency within the entire cornea is to be obtained by counts of specific stages in a wide meridional strip than by counts of all stages within a much smaller area.

The difference in frequency between the two corneas of the same animal may also be considerable. As an extreme, one rabbit cornea contained about one-third fewer dividing cells than the other cornea of equal area. This may be an exceptional condition, but in general the frequencies in the two eyes are not similar. We have not made complete counts of a large enough number of corneas to make conclusive statements, but averages obtained from a series of meridional strips indicate that ratios of frequency in the two eyes of about 1.1 to .9 are not uncommon. Buschke et al., using sample strips .77 sq. mm. in area, have detected a similar range of variability in the rat. The number of mitoses in the two eyes which they found most similar, occurred, according to our calculations, in a ratio of about 1.01 to .99, and in the most dissimilar in a ratio of 1.16 to .84, the average for nine counts being 1.08 to .92. Despite generalizations that appear in the literature to the effect that differences from eye to eye of the same animal are relatively small, most workers find occasional striking dissimilarity (cf. Kornfeld's counts on the salamander, '22). When experiments proceed on the assumption that the number of mitoses is equal in the two eyes of an animal (for example, Gurwitsch and Aniken, '28), the conclusions must be open to considerable suspicion. Some of the counts which these workers accepted as showing evidence of an effect induced by mitogenetic radiation may fall well within the limits of normal variability (Hollaender, '39).

The general conclusion to be derived from a study of the type here presented is that the range of variability in the frequency of mitosis in the epithelium of such mammals as the rat and the rabbit is too great to permit the use of one cornea as a control in determining small effects on frequency induced experimentally in the other. On the other hand, if large differences can be obtained experimentally, the epithelium of the cornea, especially that of the rat, provides in these flat preparations a readily available source for comparative studies of mammalian mitoses, as Buschke, Friedenwald and Fleischmann have shown recently.

PROPORTIONS OF CELLS IN THE DIFFERENT PHASES OF MITOSIS

In counting the dividing cells, we have grouped them as prophases, metaphases, anaphases and telophases. The limits chosen for each phase conform in general to standard cytological designation, although to accelerate the scoring process, we have selected the more conspicuous features of division, rather than the finer details of chromosome organization. Since in the corneal epithelium the mitotic spindles lie at all angles, it is evident that the earliest movements of the chromosomes to the poles will not be detected in some polar or oblique views, and these stages will be scored as metaphases. For this reason the actual percentage of anaphases will be slightly higher and the percentage of metaphases slightly lower than indicated in tables 7 and 8.

These two tables present summarized data. Detailed information concerning each sector and zone is available, but the range of variability is essentially the same as for the totals shown. The relative constancy in the proportions of the different stages from preparation to preparation permits a high degree of accuracy in comparisons based on counts of the more conspicuous stages, such as metaphases and anaphases. This greatly facilitates certain types of analysis (page 166).

TABLE 7

Proportions of different stages of mitosis in epithelium of corneas of rabbits. Actual counts, together with percentages and standard errors.

Stages of mitosis

EXPER. NO.	PROPHASES	METAPHASES	ANAPHASES	TELOPHASES	TOTALS
8-13-R	773 41.54 ± 1.14	451 24.23 ± 0.99	166 8.92 ± 0.66	471 25.31 ± 1.01	1861
8-13-L	1201 43.69 ± 0.95	630 22.92 ± 0.80	260 9.46 ± 0.56	658 23.94 ± 0.81	2749
9-21-L	731 46.06 ± 1.25	354 22.31 ± 1.04	124 7.81 ± 0.67	378 23.82 ± 1.07	1587
9-21-R	873 43.61 ± 1.11	426 21.28 ± 0.91	203 10.14 ± 0.67	500 24.98 ± 0.97	2002
Totals	3578 43.64 ± 0.54	1861 22.70 ± 0.46	753 9.18 ± 0.32	2007 24.48 ± 0.47	8199

TABLE 8

Proportions of different stages of mitosis in epithelium of corneas of rats. Actual counts, together with percentages and standard errors.

Stages of mitosis

EXPER. NO.	PROPHASES	METAPHASES	ANAPHASES	TELOPHASES	TOTALS
12-14-R	2232 40.13 ± 0.66	1156 20.78 ± 0.54	499 8.97 ± 0.38	1675 30.12 ± 0.62	5562
12-14-L	745 39.90 ± 1.13	435 23.30 ± 0.98	153 8.19 ± 0.63	534 28.60 ± 1.05	1867
12-16-R	4.38 43.80 ± 1.57	199 19.90 ± 1.26	80 8.00 ± 0.86	283 28.30 ± 1.42	1000
Totals	3415 40.51 ± 0.53	1790 21.24 ± 0.45	732 8.68 ± 0.31	2492 29.56 ± 0.50	8429

The preparations of the rat corneas were somewhat clearer than those of the rabbit, permitting identification of more of the later stages of nuclear reconstruction; and this may acount in part for the higher percentage of telophases detected in the rat than in the rabbit.

In round numbers, about 40% of the period of active cell division is represented by the prophases, about 20% by the metaphases, 10% by the anaphases, and 30% by the telophases. These proportions, although differing in some particulars, are of the same order of magnitude as those given by Wright ('25) for cells of the chick heart incubated at 37–38°C., by Picón ('33), for the epidermis of the mouse, and by Carlson ('40, table 1) for neuroblasts of the grasshopper grown at 26°C. We have not found the high proportion of metaphases (about 50%) reported by Buschke et al. for the rat cornea, and assume that their preparations have not permitted scoring of the early prophases and later telophases.

The proportions of the different phases, although indicating the relative amount of time spent in that stage, give no clue to its exact duration. We have made no effort to collect such data, but they may be secured by direct methods, such as observations on tissue cultures, or indirectly by measuring the effects of disturbances on the normal rhythm of mitosis (cf. Buschke et al.). In estimating the entire span of a cell generation, it must be kept in mind that the interphases are not scored by the methods which we have employed, and that probably the earliest prophases escape detection because of the low concentration of nucleic acid in the chromosomes during that period. The interphases and very early prophases constitute about two-fifths of the entire mitotic cycle in the neuroblasts of the grasshopper, Chortophaga, according to Carlson ('41). If similar proportions obtain in the mammalian mitotic cycle, each of the stages which we have counted will constitute a smaller proportion of the entire mitotic cycle, but the number of dividing cells will be substantially larger and the mitotic index correspondingly greater.

SUMMARY

In the course of investigations (to be reported subsequently) of the effects of ultraviolet radiation on the dividing cells of the epithelium of the cornea of the mammalian eye, a method was developed for making rapid counts of these cells in flat preparations. A preliminary survey of a series of untreated or control corneas indicated that considerable variability in the frequency of mitoses might exist within a single cornea or between the two corneas of one animal. A more extensive analysis of the range of this variability in the Albino rat and in the New Zealand White rabbit revealed that:

1. Mitoses are about 19 to 20 times as frequent in the corneal epithelium of the rat as in that of the rabbit.

2. In general, mitoses are least abundant peripherally and increase along a gradient toward the center. The gradient is steep in the large

cornea of the rabbit, slight to insignificant in the smaller cornea of the rat. Variability in the frequency of mitoses from area to area is accordingly much less in the rat than in the rabbit.

3. Counts of mitoses in a meridional strip across the entire cornea furnish a more reliable index of the frequency in the whole cornea than counts made in isolated regions. Counts made on two or more strips within the same cornea fall within the range of the sampling error, as determined by the χ^2 method. On the other hand, wedge-shaped sectors, extending from periphery to center, do not show such uniformity.

4. There may be a considerable difference in the frequency of mitoses between the two corneas of an animal. Such differences must be considered in experimental work where a treated cornea is compared with the other cornea of the same animal serving as a control.

5. The proportions of the various stages of mitosis were found to be relatively constant from preparation to preparation, so that counts made on the more conspicuous stages of mitosis, such as metaphase and anaphase, furnish under certain conditions a reliable index of the overall frequency.

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EFFECT OF ANTIGONADOTROPHIC SERUM ON CELL TYPES AND GONADOTROPHIC ACTIVITY OF THE PITUITARY GLAND[1]

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INTRODUCTION

It was first shown by Selye, Collip, and Thomson ('34) that repeated injections of gonadotrophic hormones lead to a refractory condition in which the gonads are no longer stimulated, but instead, undergo atrophy. This was attributed to the formation of an antagonist in the blood stream of the injected animal. These antagonists, or antihormones, have been prepared by numerous injections of crude pituitary extracts into animals of a species differing from the donors. After long periods of treatment large amounts of antagonistic substances are produced so that injection of blood serum from such an animal into an immature rat prevents the action of a simultaneous injection of gonadotrophins obtained from several different species. Treatment of an immature rat with antigonadotrophic serum causes atrophy of the gonads and castration-like changes in the pituitary gland, according to Meyer and Kupperman ('39), Severinghaus and Thompson ('39), and Finerty, Kupperman and Meyer ('40). The experiments to be described are further observations of these phenomena.

GENERAL METHODS

The source of the antigonadotrophic substance was the serum of male and female rabbits that had been repeatedly injected with an aqueous extract of sheep pituitary gland. The sera from the various rabbits were pooled, Seitz filtered and stored in the frozen state until used. Only serum was used which was of such strength that 1 cc. inhibited the action of that quantity of sheep pituitary gonadotrophic extract which, when administered over a period of 4.5 days to 21-day-old female rats, produced ovaries weighing 60 to 70 mg. Serum of this titer was found

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to be relatively non-specific in nature, that is, it was equally effective against the action of sheep, human, rat, and horse pituitary, prolan and pregnant mares' serum, but was ineffective against the gonadotrophic action of chicken pituitary (Kupperman, Mellish and McShan, '41). When the serum was assayed in the immature female rat against the pituitary gonadotrophic extract, the antiserum and the hormone were administered by separate injections in order to avoid any non-specific augmentation of the pituitary substance by the contents of the serum. This antigonadotrophic serum was used in all experiments to be reported except for the series of immature male rats which were treated with horse antigonadotrophic serum.

The pituitary glands removed at autopsy were immediately placed in Bouin's fluid, imbedded in paraffin and serially sectioned at 6 micra. They were stained with a modified Mallory's trichrome stain.

Quantitative and qualitative analyses of the cell types of the pars distalis were made after preparation of the microscopic slides. Quantitative studies consisted of making detailed differential cell counts of representative sections under oil-immersion at a magnification of 1350×. In most of the quantitative analyses to be described, three horizontal sections at equi-distant levels in the gland were studied. In each of the sections examined, all the cells in every tenth microscopic field were differentially counted. In each field examined the acidophiles were counted first, then the basophiles, and finally the total number of cells. From these figures the percentages of each cell type were calculated. The average number of cells which were counted in three sections of a gland was approximately 3000.

Qualitative analysis of the cytological appearance of a pituitary gland was based on a careful examination of the gland and a subjective estimation of its cellular composition.

The gonadotrophic content of fresh pituitary glands was determined by injecting a fine suspension of the glands into 21-day-old female rats. The suspension was obtained by finely triturating the tissue, adding distilled water, and flushing through hypodermic needles of progressively smaller bore. Nine injections were made over a period of 4.5 days on the basis of two per day. The suspension was diluted so that each assay animal received either 15 mg. or 25 mg. equivalent of fresh pituitary gland. The test rats were autopsied on the morning of the sixth day, at which time their ovarian weight, uterine weight, and qualitative responses of the ovaries were recorded.

A. Correlation of changes in percentage of cell types and gonadotrophic content of the pituitary gland with gonadal changes in female rats following cessation of antigonadotrophic serum treatment

Treatment of young rats for 10 days with antigonadotrophic serum produces a condition in which the gonads are like those of an hypophysectomized animal while the pituitary gland is like that of a castrated animal (Kupperman, Meyer and Finerty, '42). However, after cessation of a short time treatment of immature female rats with antigonadotropic serum, the hyperactive pituitary gland induces ovarian hypertrophy and precocious sexual development (Meyer and Kupperman, '39). This has been shown by Finerty, Kupperman and Meyer ('40), to be accompanied by a progressive reduction in the percentage of pituitary basophile cells to normal. The purpose of the following experiments is to extend and substantiate these previous observations by correlating the changes in cell types and gonadotrophic content of the pituitary gland with the rapid increase in gonadal development and activity following cessation of antigonadotrophic serum treatment.

Quantitative cytological studies were made in young rats at 2- or 3-day intervals for a period of 15 days after cessation of a 10-day treatment with antigonadotrophic serum. A second group consisted of adult female rats which had been treated for 54 days with antigonadotrophic serum. The pituitary glands of the adult rats were assayed for their gonadotrophic content and small portions of three or four glands in each group were removed and prepared for qualitative cytological examination.

Immature rats. Twenty-one female rats were injected subcutaneously from the tenth through the nineteenth day of life with 0.5 cc. per day, or a total dose of 5.0 cc. of antigonadotrophic serum. Groups of rats were autopsied on the first, third, fifth, seventh, ninth, twelfth, and fifteenth days after discontinuing the treatment. Littermate control rats were autopsied at the same age. Pituitary and ovarian weights, with the qualitative ovarian response, were noted for both the experimental and the control animals.

Adult rats. Adult female rats of approximately 200 days of age and 200 ± 15 gm. in weight received 0.5 cc. of antigonadotrophic serum per day, or a total dose of 27 cc. during 54 days. Rats of the same age and weight were selected for the control groups. Of these, one group was castrated on the first day of the experiment. The remaining animals served as untreated intact controls.

Twenty-eight of the antihormone-treated animals together with twenty-three normal and nineteen castrate control rats were autopsied on the day after the last injection. The ovaries, uteri, and pituitary glands of all the animals were carefully dissected and weighed to the nearest milligram.

Twenty-one of the antihormone-treated animals were autopsied 19 days following the last injection of serum, together with thirty-four normal and twenty-nine castrate controls. The weight of the ovaries, uterus, and pituitary gland was recorded for each of the animals.

The gonadotrophic content of all the groups of rats was determined by pooling the individual glands of each group and injecting them as an aqueous suspension into 21-day-old female rats. The material was so injected that each assay animal received a total dose of either 15 or 25 mg. of fresh pituitary glands. A small portion from the same region of the pars distalis was removed from three or four animals in each of the experimental and control groups and prepared for cytological study.

The changes in the percentage of cell types in the pituitary gland, and the ovarian weights, which were found after the antigonadotrophic serum treatment, are recorded in table 1 for the immature rats. In rats treated for 10 days with antigonadotrophic serum and killed on the day following the last injection, the weight of the ovaries was 5.5 mg. while those of littermate controls weighed 8.1 mg. The pituitary glands exhibited a picture of extreme basophilism: 29.9% of all the cells were basophiles, in contrast to 7.2% found in normal animals of the same age. The average percentage of chromophobes in these treated animals was 51.7, showing a definite decrease from the normal level of 73.1% found in littermate controls. Since very little variation could be detected in the percentage of acidophiles from the normal level during or after treatment, the increase in number of basophiles appeared to account for the corresponding decrease in chromophobes. The percentage of acidophiles during this short period of 10 days is insignificantly reduced, but indicates a trend which probably would be more apparent after a longer time. Three days following the last injection of serum there was apparently sufficient antigonadotrophic substance acting, since the ovaries weighed only 7.1 mg. and the basophiles were still 28.4%. During the next 2 days a marked stimulation of the ovaries occurred, so that the ovarian weight was 20 mg., which was greater than that of untreated littermates of the same age. The percentage of basophiles 5 days after stopping the serum injections was 20.6%. From the fifth to the fifteenth days following serum treatment, the average ovarian weight increased to 39.7 mg. while the percentage of basophiles decreased to 9.3%, which was considered to be within limits of normal variation. The basophiles in the pituitary glands at the end of the period of serum treatment were enlarged and many showed a large negative image of the Golgi apparatus, whereas those in the rats which were autopsied 15 days later were reduced in size and number, and did not show the Golgi image. Littermate controls showed increase in ovarian weight from an average of 8.1 mg. at 20 days to 14 mg. at 34 days of age and the percentage of basophiles remained at approximately 7.0%.

TABLE 1

Ovarian hypertrophy and progressive recovery of the pituitary gland following cessation of antigonadotrophic serum treatment of immature female rats.

TREATMENT	NO. OF RATS	AGE AT AUTOPSY	GONAD WT.	PITUITARY WT.	PITUITARY CELL TYPE ANALYSIS[1]			
					% Acid. (average for group)	% Baso. (average for group)	% Chrom. (average for group)	Total cells counted
		days	*mg.*	*mg.*				
Serum treated 10th to 20th day	5	20	5.5	2.8	18.2	29.9	51.7	10323
Untreated	4	20	8.1	1.9	19.7	7.2	73.1	8557
Serum treated 10th to 20th day	4	22	7.1	2.3	17.0	28.4	54.6	8741
Untreated	2	22	13.0	2.2	20.6	7.9	71.5	5004
Serum treated 10th to 20th day	3	24	20.0	3.1	18.0	20.6	64.7	7678
Untreated	3	24	11.0	3.1	21.8	8.5	69.7	6602
Serum treated 10th to 20th day	3	26	20.0	4.7	18.9	18.3	62.8	10902
Untreated	2	26	12.5	4.0	19.3	6.1	74.5	6358
Serum treated 10th to 20th day	3	28	20.7	3.3	18.3	14.8	66.9	10144
Untreated	2	28	14.0	2.9	21.9	6.7	71.4	5697
Serum treated 10th to 20th day	5	31	30.6	3.7	20.2	11.4	68.4	10740
Untreated	2	31	13.5	3.5	21.5	7.2	71.3	4429
Serum treated 10th to 20th day	3	34	59.7	4.3	19.4	9.3	71.3	9688
Untreated	1	34	14.0	3.2	22.8	7.7	69.5	3300

[1] No individual variations from the mean within any group greater than:
± 2.4% for acidophiles.
± 3.1% for basophiles.

Only qualitative estimates could be made of the percentages of basophiles in the pituitary glands of the adult rats because the cytological preparations were made from only a small portion of the same glands that were used for determination of gonadotrophic hormone content. The adult rats treated for 54 days with antigonadotrophic serum showed the same degree of basophilism exhibited by the control rats which were castrated for 54 days. The estimated percentage of basophiles in both conditions was 20–25%, in contrast to about 6–8% for the untreated intact controls. The ovaries of the serum treated rats were less than half the weight of those of the untreated rats, showing a condition of extreme atrophy. Assays of pooled glands from each group showed that the gonadotrophic content of both serum treated and ovariectomized rats was not significantly different, but in both cases greatly exceeded that of the untreated control rats. The ovariectomized rats had a slightly greater basophilism and gonadotrophic hormone content since they had not been subjected to gonadal secretions for 54 days while the serum treated animals were probably being affected by ovarian hormones during the early part of the treatment, before the onset of gonadal atrophy.

Those pituitary glands of the rats which were autopsied 19 days following the cessation of the 54-day period of antigonadotrophic serum treatment exhibited a normal or but very slightly basophilic condition and gonadotrophic content similar to that of the untreated control rats. The ovaries of these animals were slightly heavier than those of untreated controls, in contrast to their condition of extreme atrophy 19 days before. The pituitary glands of animals which had been castrated for the 73 days were extremely basophilic, and contained the "signet ring" cells, characteristic of long time castration. These glands also contained an increased amount of gonadotrophic hormone.

It is believed that the data cited in these two experiments show that in both immature and adult female rats the following events occur: Antigonadotrophic serum treatment does not interfere with the production of gonadotrophic secretions but prevents their action on the ovaries (Kupperman, Meyer and Finerty, '42; Meyer and Kupperman, '39). Consequently the ovaries atrophy and secretion of gonadal hormones ceases, or markedly decreases. The reduction in gonadal secretion allows basophilic secretion granules to be built up at a faster rate so that the percentage of basophilic cells increases at the expense of the chromophobes. When antigonadotrophic serum injections are stopped, the secretions of the extremely potent pituitary gland are no longer neutralized so that the excess secretion of gonadotrophin overstimulates the ovaries, causing them to become heavier than those of untreated littermate control rats. As the ovaries develop they begin to secrete gonadal hormones, as shown by increased development of the uteri, and over a period of 15 to 20 days cause a return of the percentage of basophiles to normal, probably by preventing the excessive formation of secretion granules so that fewer chromophobes differentiate into basophiles. Under all experimental conditions a high percentage of basophiles was accompanied by an increase of gonadotrophic hormone in the pituitary gland, supplying further evidence that the basophilic cells produce a part of the gonadotrophic complex (FSH?). When the percentage of basophiles returned to normal, the gonadotrophic content was also similar to that of untreated control rats.

B. Studies on the effect of antigonadotrophic serum treatment of female rats followed by castration, and by estrogen treatment

After cessation of antigonadotrophic serum treatment, there is a gradual change of the basophilic condition of the pituitary gland to normal. This takes place concurrently with ovarian hypertrophy and precocious sexual development in immature rats. The following experiments were done to determine whether these changes are dependent upon a gonadal secretion.

Immature female rats were treated from the tenth to the nineteenth day of life with antigonadotrophic serum, ovariectomized on the twentieth day, and autopsied on the thirty-fourth day. Control rats were treated from the tenth to nineteenth day with antigonadotrophic serum and autopsied on the thirty-fourth day of life. Another group of immature female rats was treated with serum from the tenth to the nineteenth day of life, then injected with 3 gamma of estradiol per day until autopsied on the thirty-first or thirty-fourth day.

Adult female rats which had been castrated for 54 days were injected with 3 gamma of estradiol per day for 19 days to determine whether their pituitary glands would return to a condition similar to those which had been pre-treated with antigonadotrophic serum.

The pituitary glands of the immature control rats returned to normal during the 15 days following cessation of the serum treatment and their ovaries hypertrophied. The pituitary glands of those rats which were ovariectomized as soon as the serum injections were stopped maintained their excessive basophilism which indicates that the gonads are necessary to reduce the percentage of basophiles to its normal value.

Injection of estradiol into rats as soon as antigonadotrophic serum was stopped prevented the ovarian hypertrophy which ordinarily follows cessation of such treatment. Rats which were allowed to recover without estrogen injection had ovarian weights of 58, 11,[4] and 120 mg.,

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[4] This animal had a pituitary gland which was more basophilic than others in its group.

15 days following cessation of serum injection. Those rats which received estradiol had ovaries weighing 6, 9 and 7 mg. However, in all cases whether from estrogen treated rats or not, the pituitary glands showed the same progressive reduction in percentage of basophiles as if no estrogen had been injected. Those rats which were treated with estrogen from the twentieth to the thirty-first day of life, after a 10-day treatment with antigonadotrophic serum, had an estimated 15–17% basophiles in their pituitary glands. Those which were estrogen treated from the twentieth to the thirty-fourth day of life had approximately 10–12% basophiles which was like that of the non-estrogen treated controls. Control rats which receive no antigonadotrophic serum treatment, but which were injected with 3 gamma of estradiol per day from the twentieth to thirty-fourth day of life had a normal percentage of cell types. The estrogen-treated rats in this experiment may show slightly more development of acidophiles than those which were not injected with estrogen, but no definite conclusion can be made without detailed cell counts.

The adult rats which had been castrated for 54 days and injected with 3 gamma per day of estradiol were found to contain as much gonadotrophic activity as those animals which had been castrated for the whole 73 days. However, those rats which were estrogen treated were much less basophilic than those which were untreated. Although the percentage of basophile cells was reduced, biological assay of the glands showed that their gonadotrophic content was as great, or greater, than those of untreated castrated rats. A possible explanation of this fact is that the follicle stimulating hormone was reduced along with the reduction in basophiles, but that the content of luteinizing hormone was as great, or greater than, in the castrated rats. The pituitary glands showed enough basophilism to account for some gonadotrophic stimulation with the follicle stimulating hormone, but the large size of the ovaries of recipient rats can probably be best explained by increased luteinization, possibly by luteinizing hormone secretion by the acidophiles.

It can be concluded from these data that the return to the normal condition which occurs in the pituitary gland of female rats after cessation of antigonadotrophic serum treatment is dependent upon the activity of the ovaries. If the ovaries are removed, the excess basophilism and high gonadotrophic content persist. When immature female rats which have been castrated for 10 days are injected with estradiol for 15 days, the basophilism of their pituitary glands is reduced. The gonadotrophic content, however, apparently remains about the same when the end-point of assay is the weight of the ovaries of immature rats. This may be explained on the basis of two gonadotrophic hormones, the follicle stimulating from the basophiles, and the luteinizing from the acidophiles. The reduction in basophiles and subsequent reduction of follicle stimulation may be accomplished by small doses of estrogen, but increased amounts of estrogen, and/or some other factor, possibly progesterone, are necessary for reduction in the amount of luteinizing hormone.

C. Studies of changes in the pituitary gland and gonads of young male rats treated with horse antigonadotrophic serum

An experiment was done to determine the effects of antigonadotrophic serum on the gonads and pituitary glands of immature male rats. However, the serum used was obtained from blood drawn from a pony which had been injected with extracts of sheep pituitary glands for 38 months. The antigonadotrophic effect of the serum was such that 2.0 cc. inhibited a dose of sheep pituitary gland extract which produced at least 80 mg. ovaries when assayed in 21-day-old female rats. The serum inhibited the gonadotrophic activity of sheep, rat, and human pituitary gland, prolan, pregnancy urine, and pregnant mare's serum, but not the gonadotrophic activity of horse or hog pituitary glands.

The serum was injected in progressively larger doses as the rat increased in age, so that those which were injected from the first to the tenth day of life received a total dose of 2.2 cc. of serum, and those rats which were injected from the first to the twentieth day of life received a total dose of 6.1 cc. of serum. Cell counts were made of pituitary glands of three rats in each of the groups as well as of those of control rats at 11 and 21 days of age. Group 1 consisted of male rats treated from the first to the tenth day of life with antigonadotrophic serum and autopsied on the eleventh day. Group II consisted of male rats treated with serum for the same period of time, but autopsied on the twenty-first day of life. Those in group III were treated with serum from the first to the twentieth day of life and autopsied the day following the last injection.

The autopsy data and results of pituitary cell type analysis are tabulated in table 2. Male rats which had been treated for the first 10 days of life with antigonadotrophic serum from the pony showed less development of the testes, and average weight of 23.7 mg., than untreated control rats whose testes weighed an average of 30.3 mg. These groups also showed development of the seminal vesicles and prostrate glands proportional to their gonadal weights. Serum treatment in these rats caused a marked reduction in percentage of acidophiles to an average of 12.6% as compared to 19.8% in control rats of the same age. The basophiles were affected in the same way as described for female rats: There was an increase from the average of 5.0% present in the control rats to 21.7% in the treated rats on the day following the last injection. Those rats which were treated with serum from the first to the tenth day, then untreated for the next 10 days and autopsied on the twenty-first day, showed increased testis growth, and a return to the normal pituitary cell structure, so that they were approximately the same as untreated male rats of 21 days of age. A third group was serum treated from the first to the twentieth day of life, and autopsied on the twenty-first day. These rats showed inhibited gonadal development and secretion as their testes and accessories were poorly developed in comparison with untreated male rats of 21 days of age.

TABLE 2

The effect of horse antigonadotrophic serum on the reproductive tract and percentage of cell types in the pituitary gland of immature male rats.

TREATMENT	NO. OF RATS	AGE AT AUTOPSY	GONAD WT.	SEM. VES. WT.	PROSTATE WT.	PITUITARY CELL TYPE ANALYSIS			
						% Acid. (average for group)	% Baso. (average for group)	% Chrom. (average for group)	Total cells counted
Serum treated 1st to 10th day	3	11	23.7	1.5	3.0	12.6	21.7	65.7	1587
Untreated	3	11	30.3	3.5	4.8	19.8	5.0	75.2	1568
Serum treated 1st to 10th day	3	21	176.0	17.3	39.0	23.1	7.8	69.1	1765
Serum treated 1st to 20th day	3	21	50.0	4.3	17.0	10.9	18.7	70.4	1487
Untreated	3	21	171.6	6.3	33.2	21.6	9.4	69.0	1854

Treatment of male rats with antigonadotrophic serum obtained from the horse causes gonadal atrophy and subsequent increased basophilism similar to that which is described for female rats treated with rabbit antigonadotrophic serum. The pituitary gland becomes very basophilic in both sexes and then returns to normal after cessation of serum injections in the same way. The female rats showed a slight decrease in the percentage of acidophiles, whereas there was a marked reduction in the male rats followed by a return to the normal value after cessation of treatment. There is, at the present time, no satisfactory explanation for the difference in response of the two sexes.

SUMMARY

1. Treatment of immature female rats with antigonadotrophic serum, obtained by repeated injections of sheep pituitary gland extract into rabbits, results in atrophy of the gonads and secondary sexual organs, and is accompanied by an increase in the percentage of basophilic cells in the pituitary gland. The basophilism is indistinguishable from that which follows castration for the same period of time.

2. After antigonadotrophic serum treatment of immature and adult female rats there is hypersecretion of the gonadotrophic complex of the pituitary gland which induces hypertrophy of the ovaries. During a period of 15 to 20 days following serum treatment, the percentage of basophile cells in the pituitary gland returns to normal. In the adult rats, the gonadotrophic content of the glands was reduced in those groups of rats which showed reduction of basophilism, but was high in those rats which were very basophilic.

3. The return of the percentage of cell types and gonadotrophic content of the pituitary gland to a normal condition after antigonadotrophic serum treatment is dependent upon the presence of functional gonads. Injection of estradiol reduces the percentage of basophiles at the same time that it prevents ovarian hypertrophy. Estradiol treatment of castrated adult rats reduces the percentage of basophiles in their pituitary glands but does not effectively reduce their gonadotrophic content.

4. Treatment of immature male rats with antigonadotrophic serum, obtained by repeated injections of sheep pituitary gland extract into a pony, results in atrophy of the testes and accessories, accompanied by a decreased percentage of acidophiles, and an increased percentage of basophiles. After serum treatment the testes hypertrophy and the percentages of the cell types in the pituitary gland return to normal.

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THE EFFECTS OF SIMULATED ALTITUDES UPON THE INCISOR OF THE RAT[1]

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ONE PLATE (SIX FIGURES)

A thorough review of current dental literature revealed no concrete information regarding the possibility of cellular changes in the dental tissues of humans or laboratory animals following regular exposure to altitude changes. It was hoped that the experimental work presented here might provide some information as to changes in cellular activities in teeth following prolonged exposure to low barometric pressure.

White rats were selected for the experiment. The rat incisor is particularly favorable for metabolic studies on teeth because it continues to grow throughout the life of the animal (Schour and Steadman, '35). Effects of parathyroidectomy, hypophysectomy, fluorosis, dietary deficiencies, etc., are all reflected in some way in the dentin-enamel structure of the rat incisor (Schour, '39). The effects of these changes are usually observed in the series of concentric lamellae in the dentin immediately surrounding the pulp canal. These lamellae appear in sections as incremental bands or rings which have an average width of 16 micra (the daily appositional growth of the dentin) (Schour and Hoffman, '39). Although the structure is uniform throughout, a part of this daily increment stains with eosin, and another part with hematoxylin (figs. 1, 3). The basophilic portion is believed to be that which is calcified during the daylight hours (Schour and Steadman, '35). It was hoped that changes in this complex morphology might reflect the effects of extreme altitudes.

PROCEDURE

The rats were subjected to a total of twenty-four decompressions simulating altitudes from 24,000 to 26,000 feet, 3 hours each day over a period of 25 days. Supplementary oxygen was not given during the exposure to altitude. For 13 days the rats were on a self-selected diet.

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[1] The material in this article should be construed only as the personal opinion of the writers and not as representing the opinion of the Navy Department officially.

They had access to the ingredients of a complete diet and there is no reason to suppose that they were not adequately nourished during this period. For the rest of the experiment they were on a stock diet.

The animals were segregated into male and female groups. Each decompressed animal was paired with a litter mate of the same sex as the control. The weight record is outlined in table 1. Of a large series so treated, two pairs of males and two pairs of females were selected whose weights were similar at both the beginning and end of the experiment (table 1).

TABLE 1

	C[1]	E[2]	C	E	C	E	C	E
Rat number	86	85	90	89	107	105	106	112
Sex	M	M	F	F	M	M	F	F
Weight before experiment	61 gm.	60 gm.	65 gm.	70 gm.	45 gm.	50 gm.	44 gm.	42 gm.
Weight after experiment	116 gm.	117 gm.	135 gm.	127 gm.	112 gm.	105 gm.	104 gm.	100 gm.
Average width of dentin (cross section), pulp canal to proximal at middle $\frac{1}{3}$ of root (μ)	365.8	354.0	389.4	401.2	318.6	336.5	365.8	354.0
Total width of measurable incremental rings in dentin (μ)	129	227	318	251	125	203	82	238
Total number of measurable dark and light incremental rings	8	14	8	17	8	13	5	15
Average width of dark and light (daily) incremental rings (μ)	16.1	16.2	14.8	14.8	15.6	15.6	16.4	15.9
Total of measurable dark rings	42.3	51.4	32.7	58.1	43.4	79.2	28.2	79.2
Average width dark ring (μ)	5.3	3.6	4.1	3.4	5.4	6.1	5.6	5.3
Degree of diffuseness of dark rings	††††	††	††††	†	††††	†	††††	†
Degree of sharpness of dark rings	†	††	†	†††	†	†††	†	†††
Relative number of auxiliary dark rings	†	†††	††	††		†††	††	†††
Total width of light and dark rings (μ)	86.7	175.6	85.3	192.9	81.6	123.8	53.8	158.8
Average width of light rings (μ)	10.8	12.5	10.7	11.3	10.2	9.5	10.8	10.6
Ratio $\frac{\text{dark rings}}{\text{light rings}}$	0.49	0.29	0.38	0.30	0.53	0.64	0.52	0.50

[1] C = control [2] E = decompressed

At the end of the altitude runs the rats were killed with nembutal (pentobarbital sodium) by intraperitoneal injection. They were then perfused through the aorta with formalin-Zenker solution and portions of the maxillae and mandibles were decalcified and sectioned so as to yield longitudinal and transverse sections of the incisors and molars. The sections were stained with hematoxylin and eosin. The incisors of the upper jaw were studied most intensively and the data are presented in table 1.

RESULTS

1. The decompressed rats (E) show no differences from the controls (C) in the total amount of dentin, the width of the incremental rings, the width of the light and dark portions thereof, and the ratio of all measurable dark rings to all measurable light rings.
2. The total number of measurable dark and light rings is greater in the dentin of the animals exposed to altitude than in those which were not (figs. 2, 4).
3. The most marked differences between the two groups are in the sharpness of the constituent rings and in the number of subdivisions (auxiliary rings) of the dark rings of the experimental group. The pale rings are equally subdivided in this group.

DISCUSSION

The results may be interpreted most simply as indicating that: (a) exposure of rats to altitude as in these experiments does not affect the rate of deposition of the organic matrix of the dentin, as shown by the constant average width of dark plus light rings, (b) Calcification of the dentin is disturbed, as shown by the presence of auxiliary dark and light rings, their sharpness and increased number.

The increased sharpness of the dark and light rings, with a coincidentally deeper staining of the former and paler staining of the latter, has been observed to follow the general disturbances of adult rats and is associated with endocrine imbalances, following anesthesia, and more specific calcium or dental disturbances as in fluorosis, calcium administration or withdrawal, etc. It has been referred to as the "calciotraumatic ring (Schour, Chandler and Tweedy, '37). It is assumed then, that the increased sharpness of the rings in the dentin of the decompressed rats is related to metabolic alterations associated with the daily prolonged anoxia. The subdivision of both dark and light rings into auxiliary rings has not been described before. This in all probability is another manifestation of daily exposure to anoxia.

In the pulp tissue of two rats (nos. 85 and 86) exposed to altitude decompression, but not in any of the control animals, peculiar distortions of the odontoblastic membrane were visible (figs. 5 and 6). These may be artifacts of fixation, but it is possible that they represent distortions due to the formation of bubbles. In view of the small number of animals no definite conclusions can be made as to the origin of these spaces.

SUMMARY AND CONCLUSIONS

1. Exposure of rats, in which the diet of the control animals was the same as the group exposed to altitude (24,000 to 26,000 feet for 3-hour periods), did not affect the rate of growth of the incisor teeth, as shown by the constant average width of dark plus light incremental rings in the dentin.
2. Calcification of the rat dentin, however, was disturbed, as shown by the presence of auxiliary dark and light incremental rings and by other changes in the dentin.
3. Since the decompressed rats were not suffering from malnutrition it appears that anoxia was in some way responsible for the changes in the dentin.

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PLATE 1

EXPLANATION OF FIGURES

Photomicrographs showing transverse and longitudinal sections of the rat maxillary incisors.

1 Transverse section of upper incisor of control rat no. 106, × 25.

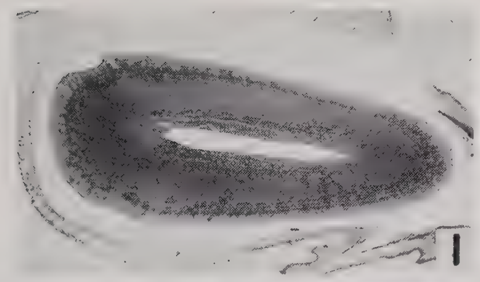
2 Transverse section of upper incisor of experimental rat no. 112, litter mate of no. 106, × 25. Note the increased number and sharpness of the dark incremental rings.

3 Higher power view of a portion of the field in figure 1, × 300.

4 Higher power view of a portion of the field in figure 2. × 300. Note that the dark and light incremental rings are more discrete than the control, and that they are sub-divided into auxiliary rings.

5 Higher power view of spaces shown in figure 6, × 58.

6 Longitudinal view of upper incisor of experimental rat no. 85, × 6. Note presence of spaces in pulp distorting the odontoblastic layer.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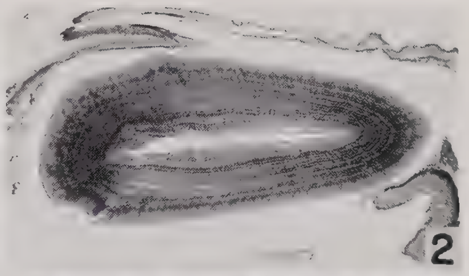
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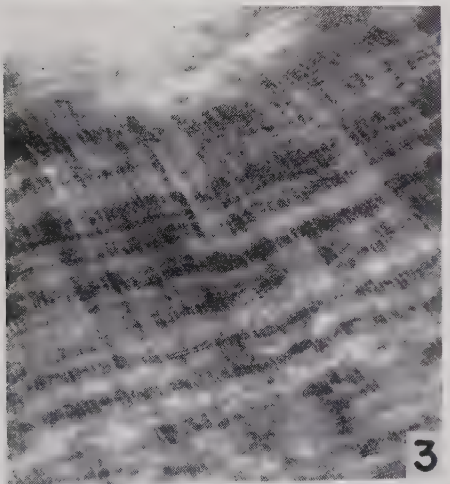
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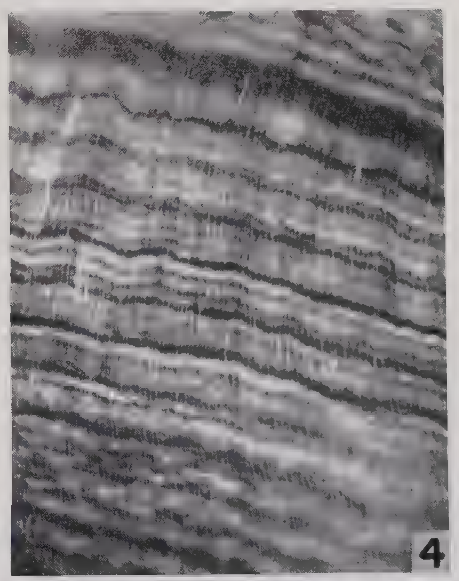
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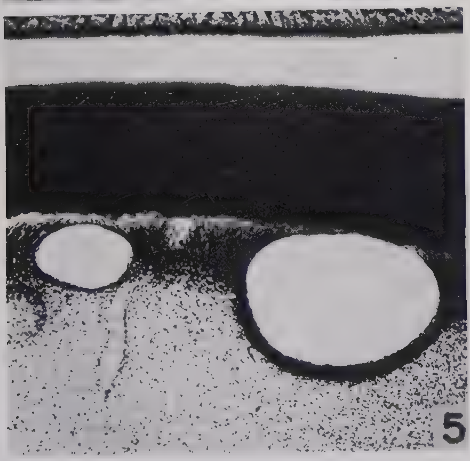
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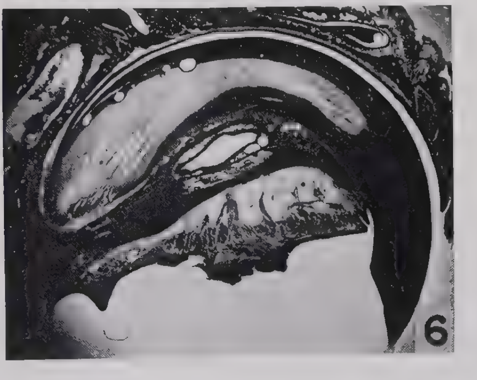
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THE INNERVATION OF THE PHARYNX IN THE RHESUS MONKEY, AND THE FORMATION OF THE PHARYNGEAL PLEXUS IN PRIMATES

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TWO FIGURES

The pattern of the nerves which innervate the pharynx in mammals has been described grossly in several forms. These descriptions usually are lacking in detail, and it appears likely that many of them were greatly influenced by the literature on man, the a priori assumption being that the condition was the same. Stimulation and degeneration experiments have established in several forms, including the macaque, the central origin of the sensory and motor neurones involved. However, the scarcity of comparative data on the pattern of the nerve trunks reaching the pharynx makes a synthesis of this problem pertinent.

For this study of the pharyngeal innervation in the rhesus monkey (Macaca mulatta), three animals were dissected in great detail with the aid of a binocular dissecting microscope. This condition is compared with that in man, in two specimens of the tree-shrew, Tupaia, and in one of Tarsius. I wish to thank Dr. Adolph H. Schultz, Laboratory of Physical Anthropology, Johns Hopkins Medical School, for the loan of specimens of Tupaia and Tarsius.

It should be emphasized that the author was not interested in the question of minute variability, and that these descriptions are not to be regarded as invariable. The anastomoses of the posterior cranial nerves probably are not identical in any two specimens of the rhesus monkey, and the great variability in this region in mammals has been mentioned by many authors.

The pharynx in man is innervated chiefly through branches of the pharyngeal plexus (fig. 2F). The latter lies on the lateral surface of the middle constrictor, and is formed by union of the pharyngeal rami of the glossopharyngeal and vagus nerves, and by laryngeal branches of the superior cervical ganglion. The several pharyngeal rami of the ninth nerve are small, and leave the trunk of the nerve near the level of the external carotid artery. The corresponding twigs of the vagus and sympathetic arise from the nodose and superior cervical ganglia respectively. The motor elements of the pharyngeal plexus supply the superior, middle and inferior constrictors, the pharyngopalatinus, the glossopalatinus, the levator veli palatini and the muscles of the uvula as well as the striated oesophageal constrictors. The palatal muscles are supplied by an ascending branch of the plexus, first described in man by Henle (1868), and later confirmed by Cords ('10). The inferior constrictor occasionally is supplied also by twigs (which may represent the so-called "middle laryngeal" nerve) from the external laryngeal nerve, or by recurrent branches of the vagus. Twigs of the lingual ramus of the ninth nerve carry sensation to the soft palate, the palatine tonsil, the palatine arches, and at least to part of the mucous membrane underlying the superior constrictor muscle. Branches of the sphenopalatine ganglion also reach the nasopharynx, and parts of the soft palate and tonsillar region.

The recent clinical evidence bearing on the function of the ninth nerve in man has been collected and discussed by Reichert and Poth ('33). Much of these data are derived from cases of glossopharyngeal neuralgia, whose treatment by intracranial section of the nerve has been described by Dandy ('27). The only somatic motor fibers in the ninth nerve reach the stylopharyngeus muscle through twigs of the lingual ramus. The pharyngeal ramus, and thus the glossopharyngeal part of the pharyngeal plexus, is entirely sensory since swallowing (i. e., action of the palate and pharyngeal constrictors) is not affected by the intracranial section of the nerve. Tactile sensation of the nasopharynx, soft palate, tonsillar region and posterior pharyngeal wall from the eustachian tube to the tip of the epiglottis, is carried by glossopharyngeal fibers. The peripheral pathways for these fibers have not yet been delineated clinically in man. Very probably they are carried by the lingual ramus of the ninth nerve and by the ascending branch of the pharyngeal plexus. Since twigs from the sphenopalatine ganglion pass into the soft palate and nasopharynx, it is possible that they carry glossopharyngeal fibers.

It seems evident that the motor fibers to the palate, pharynx and upper oesophagous originate as vagal and bulbar accessory roots, and enter the pharyngeal plexus in the pharyngeal ramus of the vagus. This branch also carries sensory neurones from the vagal roots; the bulbar accessory roots appear to be chiefly motor.

The innervation of the pharynx in the rhesus monkey differs from that of man, and from the incomplete description by Christensen, in Hartman and Straus "Anatomy of the Rhesus Monkey" ('33). It should be emphasized in the following discussion on the rhesus monkey and other forms, that when a nerve branch is said to enter a certain muscle of the pharyngeal wall, it is not to be interpreted that this branch is solely motor. Because of the uncertainty of tracing the smaller twigs by gross methods, it is often impossible to tell whether or not a sensory component is present.

The glossopharyngeal nerve in Macaca mulatta (figs. 1 and 2 D, E) divides into three main trunks in the neck distal to the jugular ganglion.

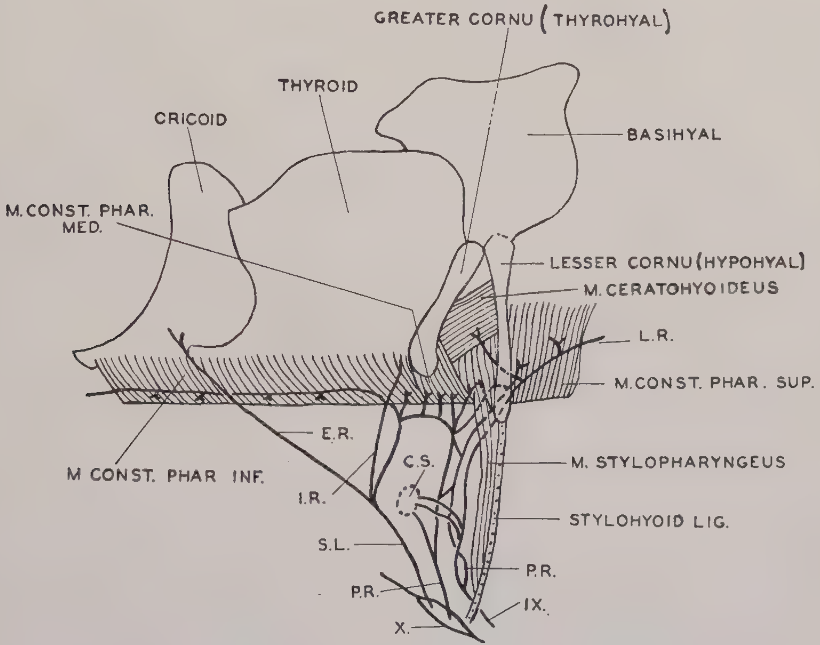
GREATER CORNU (THYROHYAL)
THYROID
CRICOID
BASIHYAL
M.CONST. PHAR. MED.
LESSER CORNU (HYPOHYAL)
M. CERATOHYOIDEUS
L.R.
M.CONST. PHAR. SUP.
E.R.
C.S.
M. STYLOPHARYNGEUS
M CONST. PHAR INF.
I.R.
STYLOHYOID LIG.
S.L.
P.R.
P.R.
IX.
X.

Fig. 1 Lateral view, semidiagramatic, of the pharynx, larynx and hyoid apparatus of Macaca mulatta, showing the pharyngeal nerves and muscles. The nerves are from the same specimen as shown in figure 2D. C.S. = carotid sinus; E.R. = external ramus; I.R. = internal ramus; L.R. = lingual ramus; P.R. = pharyngeal ramus; S.L. = superior laryngeal nerve.

These arise at about the same level, and are (1) the lingual, (2) the pharyngeal and (3) the carotid sinus rami. The lingual branch passes deep to the stylopharyngeus muscle, gives a filament to that muscle, and as it reaches the dorsal border of the pharynx sends a twig to innervate the ceratohyoideus muscle. It turns anteriorly and as it passes deep to the anterior or lesser hyoid cornu, gives several small branches which pierce the superior constrictor muscle. These probably are sensory to the mucous membrane of the anterior pharynx.

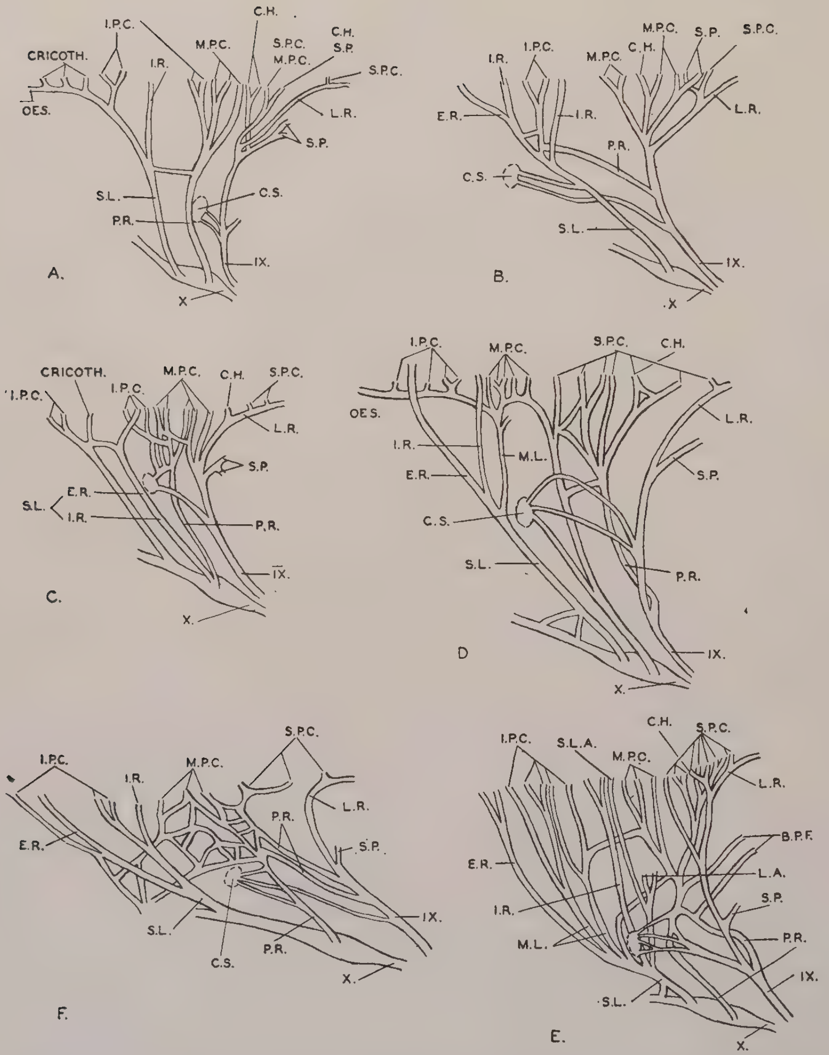
C.H.
I.P.C.
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Fig. 2 Schema of the pharyngeal nerves in Tupaia nicobarica (A), Tupaia chinensis (B), Tarsius fraterculus (C), Macaca mulatta (D and E), Homo sapiens (F). A is modified from Sprague ('44), and F from Hirschfeld and Leveille (1853). B.P.F. = bucco-pharyngeal fascia; C.H. = M. ceratohyoideus; C.S. = carotid sinus; E.R. = external ramus; I.P.C. = M. constrictor pharyngis inferior; I.R. = internal ramus; L.A. = branch to lingual artery; L.R. = lingual ramus; M.P.C. = M. const. phar. medius; Oes. = oesophageal constrictor; P.R. = pharyngeal ramus; S.L. = superior laryngeal nerve; S.L.A. = branch to superior laryngeal artery; S.P. = M. stylopharyngeus; S.P.C. = M. constr. phar. superior.

The ceratohyoid muscle, previously undescribed in the rhesus monkey, lies deep to the middle constrictor and originates from the anterior edge of the thyrohyal (greater hyoid cornu). Insertion is on the posterior surface of the hypohyal (lesser hyoid cornu).

The large pharyngeal branch of the ninth nerve passes medial to the lingual ramus, gives one or several twigs which could not be traced beyond the bucco-pharyngeal fascia, and anastomoses with the pharyngeal ramus of the vagus, which had centrally received twigs from the superior cervical ganglion From this junction a series of branches pass into the middle and superior constrictors, without however forming an extensive plexus as in man. Certain fibers were found passing anteriorly to the muscles of the palate. At least one, and in some cases two anastomosing branches connect the pharyngeal ''plexus'' and the middle laryngeal nerve.

The branch of the glossopharyngeal to the carotid sinus and body, which in one case arose from the pharyngeal ramus, was seen to ramify over the surface of the superior thyroid, lingual, and external and internal carotid arteries, after having been joined by branches of the pharyngeal branch of the vagus and the superior cervical ganglion. Boyd ('37) studied the nerve supply to the carotid sinus and body in a large series of human cadavers. He found a great constancy in the position and character of the carotid branch of the ninth nerve; it usually arose by two roots from the main trunk, or from one of the pharyngeal branches. It had various communications with the pharyngeal branch of the vagus, the superior cervical ganglion, the superior laryngeal nerve, the trunk of the vagus, and the hypoglossal trunk and its descending ramus, in this order of frequency. From the three specimens examined it appears probable that the carotid sinus nerve shows a comparable variation in the rhesus macaque.

The pharyngeal branch of the vagus nerve arises from the nodose ganglion, close to the point of entry of the communication from the accessory nerve. After contributing several twigs to the intercarotid plexus, it joins the glossopharyngeal nerve as described above.

The superior laryngeal nerve arises distal to the nodose ganglion, receives communications from the superior cervical ganglion, and breaks up almost immediately into a number of trunks (fig. 2, D and E). The two largest of these are the external and internal rami, which enter the larynx in company with the superior and inferior laryngeal nerves as in man. Between these two rami are a variable number of branches which enter the inferior constrictor muscle, and which receive communications from the pharyngeal rami. These branches probably represent the so-called "middle laryngeal" nerve, first described by Exner (1884) in the dog. It is found in many mammals, but usually is stated to be lacking in man. Lemere ('32) encountered it in one-half of the dogs studied, and found it variable in position, originating from the pharyngeal plexus, the external ramus of the superior laryngeal, the nodose ganglion, the main vagus trunk or direct from the bulbar roots. In many dogs and in man, its presence is probably obscured in the extensive pharyngeal and laryngeal plexuses, although it is occasionally distinct in man as a branch of the external ramus. Its origin from the superior laryngeal nerve appears to be reasonably constant in the rhesus monkey.

Anterior to the internal branch of the superior laryngeal nerve in one specimen of the rhesus monkey is a branch which gives twigs to the superior laryngeal and lingual arteries. The inferior constrictor muscle may or may not receive fibers from the external branch of the superior laryngeal nerve, and in one case was supplied together with the striated oesophageal constrictor by a descending ramus of the middle laryngeal nerve (fig. 2D).

The nodose ganglion receives communications from the eleventh and twelfth cranial nerves, and from the superior cervical ganglion; no anastomoses with the ninth nerve were observed. The lower end of the superior cervical ganglion gives twigs to the junction of the pharyngeal rami of the ninth and tenth nerves, to the intercarotid plexus, to the superior laryngeal nerve, and to the first two cervical nerves. Riegele ('26) found rami communicantes from the superior cervical ganglion to the first three cervical nerves in M. mulatta, and van den Broek ('08) observed twigs connecting this ganglion with the ninth, tenth and twelfth nerves in M. irus. Zuckerman ('38) has described the sympathetic system in detail in M. mulatta.

The anatomy of the cervical portion of the hypoglossal nerve will be described because of its interesting anastomoses, although it does not demonstrably contribute to the innervation of the pharynx. Central to the descending ramus of the hypoglossal, one or three stout branches are received from the first and second cervical nerves, similar to those described by Fiendt ('14) and by Corbin, Lhamon and Petit ('37). From the hypoglossal trunk, peripheral to the entrance of the cervical branches, a long slender twig is given off to the sternocleidomastoid muscle in two of the animals dissected. Such a branch requires further comment.

Neither Beevor and Horsley (1888), Sternberg (1898), Sherrington (1898), Howell and Straus ('33), nor Corbin and Harrison ('39) has found evidence of motor fibers contributed by the first cervical nerve to M. sternocleidomastoideus in the rhesus monkey. Corbin, Lhamon and Petit ('37) have described dorsal root ganglia on the first cervical nerve in three out of fifteen rhesus monkeys. It is possible that in such specimens, this nerve furnishes sensory fibers to the sternocleidomastoid muscle via the hypoglossal trunk. Unfortunately the roots of the first cervical were not examined in this study.

The ramus descendens hypoglossi receives branches only from the second and third cervical nerves. In one animal a slender branch extended posteriorly from the nodose ganglion, and anastomosed with two of the infrahyoid branches of the ansa hypoglossi, and with the third cervical nerve. Fibers from this vagal branch seemed to pass into both bellies of the omohyoid muscle in company with the ansa branches. The branch to the sternocleidomastoid, and the anastomoses with the vagus are hitherto undescribed in this form. The cervical communications of the hypoglossal nerve do not agree with the results of Huntington (1897), Tanaka ('32) and Cordier et al. ('36). Fiendt found in one specimen a contribution from the fourth cervical nerve. Beevor and Horsley (1888) and Sherrington (1898) have demonstrated by stimulation experiments the muscular field supplied by the descending branch of the hypoglossal and its cervical contributions.

The pharyngeal nerves of macaque monkeys have been stimulated by several workers. The most important of these contributions are by Beevor and Horsley (1888) in Macaca radiata, and by Rethi (1893) in M. sp.? and Kreidel (1897) in M. mulatta. Although none of these workers has described the pattern of the pharyngeal nerves, it is certain from their experiments that the motor fibers of the lower vagus and the bulbar accessory roots reach the palatal and pharyngeal muscles (with the exception of Mm. tensor veli palatini and stylopharyngeus) exclusively by way of the pharyngeal ramus of the vagus. The stylopharyngeus may be supplied by the lingual branch of the glossopharyngeal (Beevor and Horsley), or through the pharyngeal ramus of the vagus (Rethi). The ceratohyoid was not mentioned in these experiments.

There has been considerable controversy as to whether the tensor veli palatini is innervated by a branch of the trigeminal nerve, or by the bulbar accessory through the pharyngeal plexus. This argument has been reviewed and discussed by many authors, including Turner (1889), Edgeworth ('13) and Rich ('20). It appears certain through experimental and developmental studies that the classical viewpoint of trigeminal supply is correct.

The studies of Beevor and Horsley, and of Kriedl indicate that the cricothyroid is supplied by the external ramus of the superior laryngeal nerve in Macaca. The superior laryngeal apparently was entirely sensory in the monkeys studied by Rethi, the cricothyroid being supplied by fibers carried in the pharyngeal ramus of the vagus. The superior laryngeal is entirely sensory in the cat according to Ranson, Foley and Alpert ('33). Lemere ('32) found that in the dog, the external laryngeal ramus sometimes carried motor fibers to the inferior constrictor as well as the cricothyroid; this is apparently also the condition in man. Botar, Popjak and Bense ('37) have made a comparative study of the fiber content of laryngeal and pharyngeal nerves in the cat, dog, monkey (sp. ?), chimpanzee and man.

The present study has shown that the inferior constrictor muscle in the rhesus monkey is supplied solely by branches of the superior laryngeal nerve, which probably correspond to the middle laryngeal branch of certain other animals. In view of the experimental findings discussed above, these motor fibers must pass into the middle laryngeal by way of the large anastomosing branch from the pharyngeal ramus of the vagus.

Comparative data

It seems very probable from dissections of the rhesus monkey (Macaca mulatta), two species of oriental tree shrews (Tupaia nicobarica, and T. chinensis), the tarsier (Tarsius fraterculus), and from the literature on the baboon (Papio) and the orang (Pongo pygmaeus), that man represents a culmination of increasing plexus formation of the pharyngeal region in primates. Furthermore, there has been a shift in the pattern of the nerves supplying the pharynx, accompanying the formation of the plexus.

It has been shown (Sprague, '44) that there is no discernible pharyngeal plexus in Tupaia. In one specimen of T. nicobarica (cf. Sprague, '44), the glossopharyngeal nerve had no pharyngeal ramus but supplied the pharyngeal wall anterior to the middle constrictor by twigs from the lingual ramus (fig. 2A). The pharynx posterior to the middle constrictor was innervated by the pharyngeal branch of the vagus (which however had no anastomoses with the glossopharyngeal), and by the external ramus of the superior laryngeal nerve. A large anastomosis was present between these two vagal branches. A specimen of Tupaia chinensis, dissected in the course of the present study (fig. 2B), had on the contrary, no pharyngeal branch of the vagus, as such, but showed however, a prominent pharyngeal ramus of the glossopharyngeal, which joined the superior laryngeal nerve. Branches from this junction supplied the pharyngeal wall posterior to the middle constrictor, and also the striated oesophageal musculature. Twigs of the lingual ramus of the glossopharyngeal nerve innervated the pharynx anterior to the middle constrictor. One of the two branches of the external ramus of the superior laryngeal nerve pierced the middle of the thyroid cartilage.

Thus, in Tupaia, there is no pharyngeal plexus, and although there is some variation in the peripheral distribution, there is a partial or complete separation of glossopharyngeal and vagal fields. The middle constrictor muscle appears to be the dividing line, although both nerves contribute to the innervation of this level. While it has been shown that in man and macaque, the motor fibers to the pharyngeal wall are derived from vagus and bulbar accessory roots (except for M. stylopharyngeus and probably also M. ceratohyoideus), it appears likely that many of these fibers are glossopharyngeal in origin in Tupaia.

A dissection of Tarsius fraterculus (fig. 2C), disclosed a condition similar to that in Tupaia chinensis, except for a slight plexus formation in the branches of the pharyngeal ramus of the glossopharyngeal. The superior laryngeal nerve arose from the main vagus trunk in two branches in the specimen dissected.

The results of the careful work of Riegele ('26) are somewhat obscured by the great detail of his plates, which however seem to indicate a pharyngeal plexus formation in the baboon (sp.?) and the orang similar to that in the macaque, but somewhat more extensive.

Eisler's (1890) studies on the gorilla show that in the peripheral distribution of the pharyngeal nerves, this animal is very similar to man. A considerable pharyngeal plexus is present.

It is obvious from the results presented here, that very considerable differences in the pharyngeal innervations are present in various types of primates, and of other mammals. This region, rather difficult of access, has frequently been assumed and described by various authors as similar in any particular form to that in man.

DISCUSSION

The pharynx in man is innervated chiefly through an extensive pharyngeal plexus formed by pharyngeal rami from the ninth and tenth nerves and from the superior cervical ganglion. There may be some contribution to the inferior constrictor from the external branch of the superior laryngeal nerve. This pharyngeal plexus lies chiefly on the lateral surface of the middle constrictor muscle, and ascending and descending branches are given off to the palate and the striated muscles of the oesophagous respectively.

It has been shown that in primitive forms as Tupaia and Tarsius, the pharyngeal ramus of either the glossopharyngeal or the vagus may be missing. The pharyngeal ramus present anastomoses with the superior laryngeal nerve, the latter aiding in the distribution of pharyngeal fibers. As the terminal twigs to the pharynx become organized into a plexus in more specialized primates, the superior laryngeal nerve is utilized less and less as a pathway for motor and sensory neurones to the pharynx, and become dissociated from the pharyngeal rami. The latter become constant and distinct entities, and take over the nerve supply of the pharynx. The external branch of the superior laryngeal nerve continues frequently to contribute to the innervation of the inferior constrictor, and to anastomose with the pharyngeal plexus. The well-developed pharyngeal plexus, as seen in man, is usually said to be formed by pharyngeal branches of the ninth, and tenth nerves and the superior cervical ganglion. Phylogenetically, however, the superior laryngeal nerve also contributes to the plexus, as seen in Tarsius and Macaca, and frequently also in man.

These conclusions are not meant to apply to animals other than primates. Lemere ('32) has shown that an extensive pharyngolaryngeal plexus is present in about one-half the number of dogs examined, and a similar condition probably is present in the cat and many other carnivores, and perhaps in other orders of mammals. The present series of primates dissected is not large enough to preclude the possibility that exceptions to this trend may be found, but the trend itself appears to be present in the group.

The general concept, expressed by Fürbringer, of the great constancy between peripheral motor trunks and striated muscles, has been abandoned in its most rigid application by the majority of recent workers. While the general value of the Fürbringer hypothesis has been abundantly proved, the absolute dependency placed by many authors on this muscle-nerve relationship in determining homologies has resulted in a measure of confusion, and has violated the dynamic concept of individual variation of organisms. The important factor in this muscle-nerve constancy is not the gross nerve trunk, but the fibers carried within it, and their central connections. It has been shown repeatedly by both gross and experimental morphologists, and is again emphasized in the present paper, that the peripheral nerve trunks are variable, especially in certain areas of plexus formation. The available evidence suggests that there is great constancy in the specific relationship between motor nuclei (and thus also peripheral motor fibers) and striated muscle. But these fibers may, and often do, follow different nerve trunks from the central nervous system to the periphery. These statements have not in any way barred cognizance of the remarkable stability and conservatism of the peripheral nerve trunks, but they have intended to add emphasis and evidence to the modification of the concept of muscle-nerve specificity which has been growing steadily in recent years.

SUMMARY

1. The innervation of the pharynx in the rhesus monkey differs from that in man in that a well-formed middle branch of the superior laryngeal nerve is present, and that the pharyngeal plexus is poorly developed.
2. The motor fibers to the pharynx and palate in the macaque leave the medulla by the bulbar accessory and lower vagus roots, and pass to the musculature through the pharyngeal ramus of the vagus, except for the fibers to Mm. stylopharyngeus and ceratohyoideus which are glossopharyngeal in origin, which are in some cases distributed through the lingual ramus of this nerve. In Tupaia and Tarsius, it is probable that some of the motor fibers to the superior and middle constrictors also leave the brain stem via glossopharyngeal roots.
3. It appears, from a comparative study of Tupaia, a primate-like insectivore, and Tarsius, a primitive primate, together with the rhesus monkey, that a trend of increasing plexus formation of the pharyngeal nerves is present in primates, culminating in man. No pharyngeal plexus is found in Tupaia, very little in Tarsius and Macaca, more in the baboon and the orang, and an extensive one in man.
4. Correlated with a lack of a pharyngeal plexus is a more or less complete separation of the vagal and glossopharyngeal fields in the wall of the pharynx. The level of the middle constrictor is the dividing line. The superior laryngeal nerve apparently is partly pharyngomotor in those forms lacking a plexus.
5. It has been shown that all primates and other mammals do not have a pharyngeal plexus as in man, and the common practice of assuming a detailed similarity of this region to that in man, is erroneous.

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ANOMALIES OF THE CARDIO-PULMONARY CIRCUIT COMPENSATED WITHOUT A DUCTUS ARTERIOSUS

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FIVE FIGURES

The degree of cardiac malformation compatible with life, and the mechanism of compensation, are questions of both theoretical and practical interest. We present a case illustrating both of these points of view.

The subject of our necropsy was a white girl who died at the age of 14 months. The mother, who had older children, considered this child normal until she was 10 months old. Her clinical history will probably appear elsewhere and will be omitted here. She was brought to the Emergency Room moribund and went on to exitus soon after.

General. The pertinent findings at autopsy were: She is a well nourished child 74 cm. long and weighs 14 K. Her digits are clubbed and her nail beds and mucous membranes are cyanotic. On the lateral aspect of her right arm there is a necrotic lesion 3 cm. in diameter.

Regional. The pleural cavities contain neither fluid nor adhesions. The heart is relatively large and lies almost transversely, filling the lower chest.

Figure 1. The crown of the diaphragm reaches the fourth rib on the right and the fifth rib on the left. The thymus weighs 3 gm. and is not enlarged. The aorta shows no anomalies of its larger branches and the bronchial arteries, coming directly from the aorta under the arch, have lumenal diameters of 1 mm. The heart weighs 70 gm. The pericardium is smooth and the coronary veins are large and prominent. Because of its almost transverse position in the body the relation of heart and lungs is abnormal.

Figure 2. The left lung drains by two widely separated pulmonary veins into the left atrium while the right lung drains through a single large venous tube just above the atrium to the right of the pulmonary artery. The right vein reaches the middle of the right atrium. A very incomplete interatrial septum arches inward from the dorsal wall near the midline leaving a large foramen. The venae cavae are nearly in the midplane of the trunk which crosses the cardiac axis at a considerable

angle. This results in the superior vena cava passing obliquely over the septum to the left atrium while the inferior vena cava passes obliquely upward to the right. The azygos vein enters the right atrium just below the superior vena cava while the coronary sinus shows a very large opening to the right of the septum just below the inferior vena cava. All of this lies over what appears to be a single left ventricle.

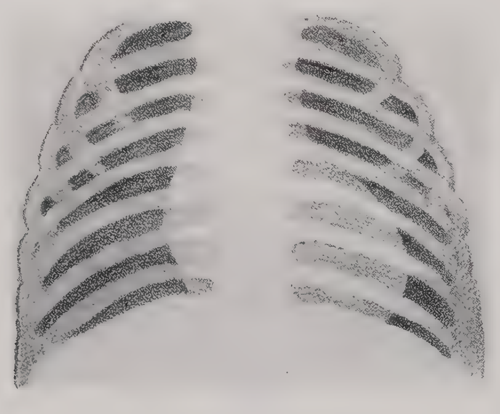

Fig. 1 Radiograph showing axial deviation of the heart.

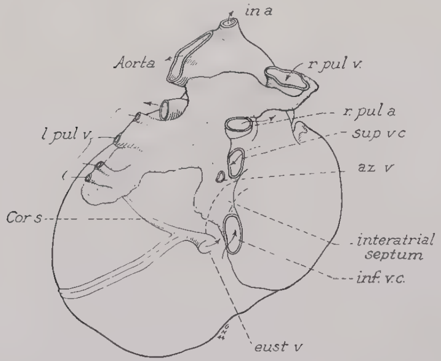

Fig. 2. Simplified dorsal view of the heart in situ.

Figure 3. The walls of this ventricle vary in thickness from 10 mm. at the base to 5 mm. at the apex. The bicuspid valve is large and well developed but it is evident that the ventricular cavity arches considerably behind the valve. From the anterior wall the aortic arch rises and branches normally.

The pulmonary arch comes from the right margin obliquely across the aorta and spirals around it to branch right and left.

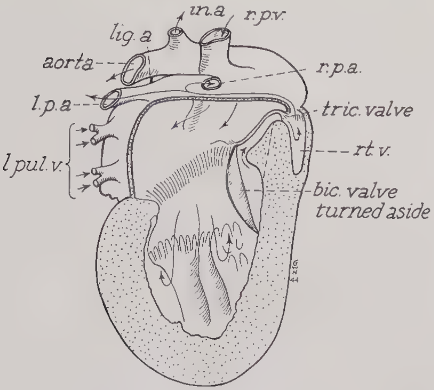

Fig. 3. Diagram of the right and left ventricles

Figure 4. Just above the origin of the left branch there is a trace of ligamentum arteriosum about 1 mm. in thickness, identifiable only by minute pits in the roof and floor of the arterial trunks. Tracing the pulmonary arch downward it shows small but well developed valves at the entrance to a small but muscular cavity lying against the upper right surface of the left ventricle. The muscle varies in thickness from 2–3 mm. The cavity is exposed in figure 4. The surface is clean and normal. Against the dorsal wall of this right ventricle is a small opening apparently completely plugged by miniature chordae tendineae which spread to the ventricular wall. A probe passes readily at the lower margin of this tricuspid valve and enters the left ventricle through an opening of approximately 2 mm. diameter. On reflecting a leaf of the bicuspid valve, as in figure 3, the cavity is found to pass into a funnel for nearly an inch to reach this opening. There is, therefore, an incomplete interventricular septum. The tricuspid rudiment can be traced to the floor of the right atrium, ending in a pit.

Microscopic examination. The myocardial parenchyma shows fading striations and occasional vacuolization of the sarcoplasm. Myocardial stroma is edematous. The endocardial cells are swollen with some round nuclei. Here also are some areas of edema.

The liver sustained an early central lobular ischemic parenchymal atrophy. The central cells are shrunken and vacuolated. The hepatic cords of the periphery of the lobule have undergone an albuminous cytoplasmic degeneration. This indicates a terminal toxemia.

Pulmonary arteries in the lung substance show, even grossly, an excess of connective tissue adventitia. Microscopically, the large veins are found to be full of infected thrombi and the arteries are full of blood. There is, however, no distinction between pulmonary and

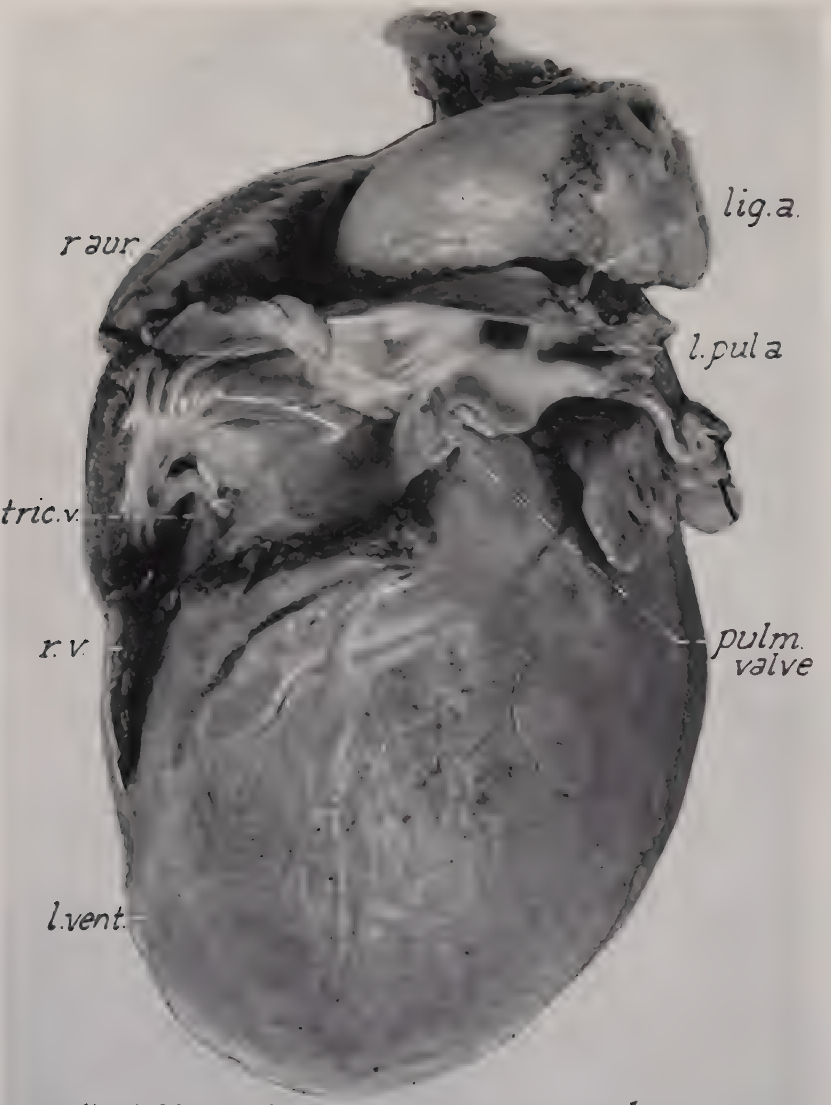

Fig.4 Ventral view on anomalous heart

bronchial arterial systems because they communicate freely. The bronchial arteries at the hilus are large (1 mm. lumen), and filled with blood, but it is difficult to find a trace of bronchial arteries out in the lung.

Figure 5 shows the relative sizes of bronchial and pulmonary arteries. The peripheral pulmonary system is normal except for excess adventitia and, in some vessels, small thrombi and intimal thickenings of recent origin. The larger bronchi show catarrhal inflammation but the bronchioles are not involved. Pulmonary epithelium is distinct and in scattered areas shows cuboid form. Some cells are rounding off and some lie free in the alveoli, together with extravasated red cells.

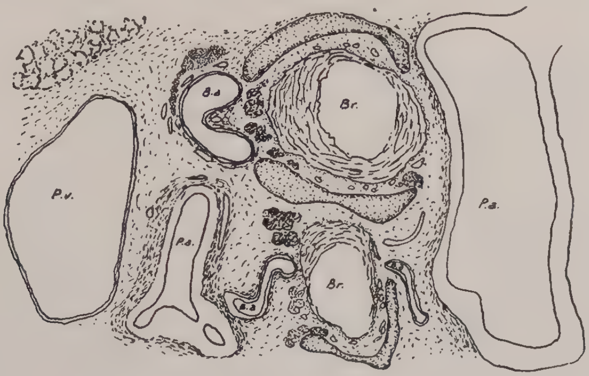

Fig 5. Hilus of lung showing large bronchial artery.

MECHANISM OF CIRCULATION AND ETIOLOGY

The course of the circulation is clear. Blood entering either pulmonary or systemic veins poured into a common ventricle from which most of it was sent to the aorta. A small proportion, under heavy pressure, was injected into the right ventricle and sent to the pulmonary circuit. It received additional bronchial arterial blood sufficient to provide a relatively large pulmonary return to the heart. The arrangement apparently worked until the appearance of infected thrombi embarrassed venous return.

Speculation as to origins of the anomalies point to a very early developmental stage. The positions of the venae cavae with respect to the interatrial septum indicate that the malposition of the heart was already present at the 10–15 mm. stage or earlier. This secondarily determined the position of pulmonary veins as atrial outgrowths to the adjacent pulmonary plexus since the tracheal bifurcation was high relative to the heart base. Both of these factors kept the interatrial septum rudimentary as the heart grew. Arrest of the growth of the right ventricle and tricuspid valve came later but still in early fetal life. It must have begun before the interventricular septum finally closed because a 2-mm. opening was left. Lack of ventricular growth indicates a lack of need for its function and that some other source of arterial blood was reaching the lung in large enough quantities to inhibit the pulmonary flow. One might suspect a ductus arteriosus but, if the rudimentary ligament is testimony, that channel must have been closed long before birth. In fact, its early stenosis indicates equalization of pressure and stasis early in fetal life. Certainly the veins were returning much more blood than the right ventricle was sending out. We do not know where the largest anastomosis occurs but small ones are numerous in the hilus of each lung.

Since the pulmonary arterial supply and bronchial anastomosis are both very restricted, pressure in the alveolar bed was probably not very high until the veins became blocked. This finally resulted in extravasation of some red cells. The unusual development of pulmonary adventitia is not explained unless it be that the dilated arteries compressed this adventitia during life while constriction revealed it after death. The arteries are not very elastic. The more peripheral intimal changes are not marked and may not be significant. Atrophic changes in heart and liver are of recent origin. The clubbed fingers indicate that the mixed blood was insufficient even though left ventriclar pressure was adequate.

THE GENERAL PROBLEM OF PULMONARY INSUFFICIENCY

Hardly any two cases in the literature are precisely alike and only a tabulation could profitably compare them. Some thirty-five cases have been studied and of these very few have become adults. The clinical identification of combined defects is very difficult. We will summarize without specific reference and review embryological principles.

Brown in 1913, MacCready in 1918, and Huntington in 1919 carefully developed the idea of a capillary plexus covering the lower foregut. This is supplied directly from the aorta (esophageal and bronchial), and later from the sixth arch (pulmonary artery). The ductus is selected out of several possible aortic channels. The area is drained by short channels to the sinus venosus (common hepatic) and later, when trachea and esophagus separate, by pulmonary veins extending from the atrial wall. Direct or indirect arterial branches therefore are related to pulmonary tissue very early. The terminal bronchial-pulmonary connections are always retained and because of pressure differences account for an appreciable share of the pulmonary capillary supply. Larger branches are unusual. Miller located such anastomoses. The early plexus is present at 4.5 mm. and the pattern is well defined by 10 mm. The separation of aortic and pulmonary trunks and valves is completed together with the interventricular septum by about 17 mm. By this time all venous entrances to the atria are complete.

In right-sided insufficiency, partial or complete, mechanical problems must be solved if life is to continue. The blood volumes in the two circuits must be balanced either internally or externally. If the tricuspid valve is small or the pulmonary artery reduced the ventricle will also remain small and the foramen ovale usually remains open. In general, the lung gets its auxiliary supply by the ductus arteriosus. If bronchial anastomosis can equalize the pressure and produce stasis in the ductus it stenoses. If both of these fail to supply the lungs with blood at birth, death follows shortly thereafter.

A modification is introduced by an incomplete interventricular septum which permits different volumes to the two circuits but under a pressure approximating that of the strongest ventricle. If this is combined with a complete interatrial septum the two streams may differ markedly in oxygen content. Those individuals who have lived to 20 or 30 years of age had no ductus arteriosus but a bronchial arterial supply, a complete interatrial septum, and a defective interventricular septum. All others are relatively short lived because their compensatory devices do not grow with the demands of the body. A large number have anomalies of normal asymmetry in heart, arches, or in other viscera. Aside from these and the direct results of mixed blood or low pressure no other anomalies are correlated with this group.

CONCLUSION

A fair degree of health and growth was attained by an infant for 14 months after birth in the face of complete interatrial mixture of pulmonary and systemic blood, a very small output from the right ventricle and bronchial anastomosis in both lungs. There was no ductus arteriosus. Death was not caused by the anomalies though they were responsible for vascular insufficiency and clubbing of the fingers.

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THE RELATION OF LYMPH FLOW TO ENDOCRINE ACTIVITY IN THE GUINEA PIG OVARY

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ONE FIGURE

The relation of the lymphatic flow to functional activity in the guinea pig ovary would seem to shed some light on the part which the lymphatic circulation may play in the transmission of ovarian hormones. This investigation was conducted to determine whether any such correlation exists. On the basis of specific anatomical changes in the lymphatic vessels surrounding follicles and corpora lutea, Anderson ('26) has suggested that the lymphatic vessels of the ovary are concerned with the transport and removal of endocrine secretion. If the lymphatic flow is concerned with the removal of hormones then the rate of flow should be proportional to the activity of the endocrine elements within the ovary. No physiological experiments have been conducted to ascertain the actuality of this supposition.

METHODS

In this experiment pregnant and non-pregnant guinea pigs were used and the lymphatics were demonstrated by injecting India ink into the ovaries. Preliminary experiments showed a leash of three to five approximately parallel lymphatic vessels emanating from the ovary. These united at a distance of about 1–2 cm. from the hilum to form one or two larger vessels which extended to the aortic lymph glands in the region of the kidney pelvis.

The India ink was filtered through a Whatman 52 filter and diluted with an equal quantity of distilled water. It was then injected into the ovary through the surface opposite the hilum, with a tuberculin syringe and a 17-gauge short bevel needle. The injection was continued until the ink had reached approximately 1.5 cm. from the hilum of the ovary. The ink was then milked along the vessels towards the aortic glands and the time taken for spontaneous refilling of the lymphatic vessels for a distance of 1 cm. was recorded. The time was observed only after spontaneous filling, thereby eliminating the effect of massage and direct pressure on open lymphatic vessels. The duration of pregnancy was estimated by the crown-rump length of the fetus. Litters of the same age were not necessarily of the same length. Nevertheless this variation was not a significant factor in the investigation. The number of pregnancies in each horn of the uterus did not affect the rate of flow in the corresponding ovary.

In those ovaries in which a lymphatic flow was shown it was possible to hasten the flow greatly by gentle massage of the ovary. In some instances a flow of lymph containing India ink was obtained only as long as pressure was being applied to the syringe and when this slight pressure was reduced the lymph flow appeared to cease completely.

RESULTS

Of the seventy-four guinea pigs which were used twenty-nine were pregnant. Of the non-pregnant, seven were post-partum and still showed placental sites. In each of these groups some ovaries could not be injected sufficiently for the lymphatic plexus leaving the hilum to fill despite increased pressure in the syringe or alteration in the position of the needle within the substance of the ovary. This condition was encountered in those ovaries which were smaller than average and where follicular growth was minimal. In each of the three groups of ovaries, namely the ovary of pregnancy, the ovary of the mature non-pregnant animal, and the ovary of the puerperium, there were those in which a lymph flow was established and those in which no flow could be shown.

	OVARY OF PREGNANCY	NORMAL CYCLE	PUERPERIUM
Total	58	80	14
With flow	28	51	9
Without flow	30	29	5

The distribution among the pregnant animals in relation to the duration of pregnancy was striking.

DURATION OF PREGNANCY (MM. LENGTH)	10	20	30	40	50	60	70	80	90
Total no. of ovaries	10	—	4	2	—	8	6	12	16
No. with flow	7	—	3	1	—	4	2	4	7
No. with no flow	3	—	1	1	—	4	4	8	9

From this table it can be seen that in the early pregnancies a lymph flow could be produced with a high degree of consistency whereas in the latter half of pregnancy the probability of demonstrating a lymphatic flow was relatively remote.

Half of the ovaries of post-partum animals showed lymphatic flow.

The rate of flow in relation to duration of pregnancy is shown on the accompanying chart. It is shown that flow from the non-pregnant ovary does not exceed on an average, a rate of 20 seconds per centimeter, whereas the flow from the ovaries of later pregnancy varies from 1–4 minutes per centimeter. In the young pregnancies the flow rates do not differ very noticeably from those of non-pregnant. When the embryo has reached a length of at least 60 mm. the rate of flow is much slower; furthermore, it must be remembered that in late pregnancy there is a greater number of ovaries in which no flow is elicited.

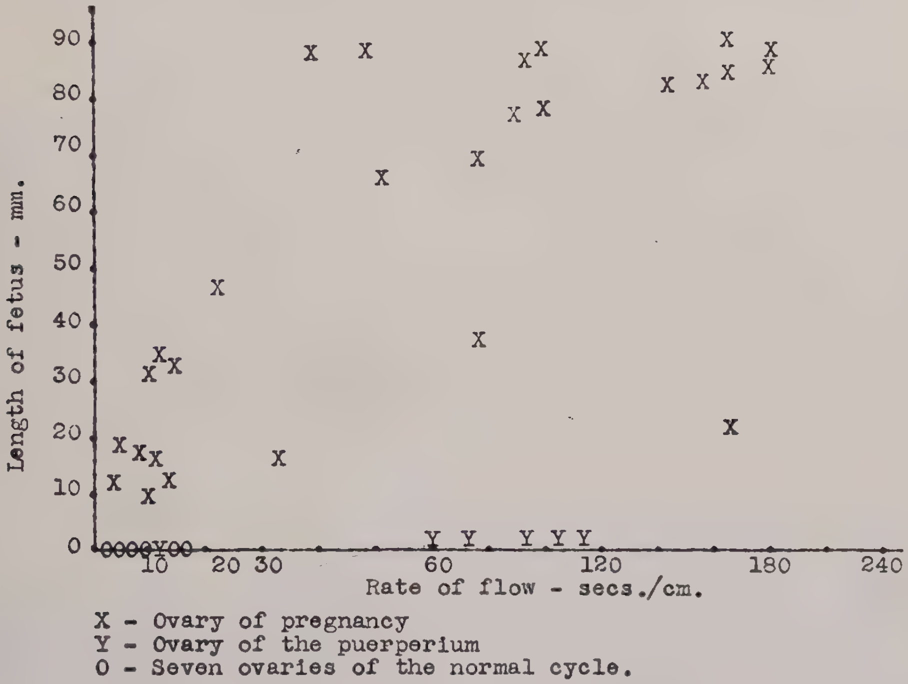

Fig. 1 Showing (a) the rate of lymph flow from the ovary in relation to the duration of pregnancy and (b) the range of flow rates in non-pregnant guinea pig ovaries.

The flow in ovaries of post-partum animals is also indicated on the chart. Their numbers are too few to warrant any definite conclusions. For the most part the range of flow in these ovaries is from 1–2 minutes per centimeter.

In certain instances the ovary on one side was small, contained few or no follicles, no corpus luteum and no demonstrable lymphatic flow whereas the ovary of the other side contained numerous follicles and/or a corpus luteum and a demonstrable lymphatic drainage.

In some of the animals no flow could be observed in either ovary. These ovaries were usually small and contained few if any macroscopic follicles.

The results of this experiment indicate that the extent of lymphatic drainage of the ovary parallels its presumed endocrine activity and subsequently as the ovarian secretion in the latter half of pregnancy wanes, the rate of lymph flow through the ovary diminishes. This relationship is compatible with the observation that the lymphatic flow is directly concerned with the transport of ovarian hormones, and that where the activity of the ovary is reduced or absent the rate of lymph flow through the ovary is minimal.

DISCUSSION

From this experiment it is noted that the flow rates in the ovaries of pregnant and non-pregnant guinea pigs differ according to the apparent physiological state of the ovary. This in turn is associated with obvious anatomical alterations in ovarian structure.

The efficaciousness of dye substances in studies upon lymphatic circulation has been substantiated by others (Landis, '34, Field and Drinker, '36). When the ink is injected into the tissue spaces a local increased permeability of capillary walls occurs and the ink appears within the lymphatic circulation very shortly afterwards.

It was observed that in the ovaries in which there was no lymph flow there were few or no follicles or corpora lutea. The relationship of follicular maturation to lymphatic flow from the ovary has been suggested by the work of Anderson. She states that in each cycle, with the maturation of follicles there is a growth and development of the vascular elements in the stroma. These vessels proliferate in the theca of the follicle and very shortly afterwards the lymphatic vessels permeate the ovarian stroma more or less evenly and as the follicle begins to grow beyond the ripening phase the lymphatics permeate the theca interna. They then reach the stratum directly beneath the granulosa layer and there form a single layered capillary network. The development of lymphatic vessels progresses as the follicle matures. When ovulation takes place the lymphatics follow the blood vessels as they invade the granulosa cells, but again lag behind in their rate of growth.

There is no apparent advancement in the growth of lymphatics in the first 2 days of the corpus luteum but the vascular capillaries have already invaded the granulosa about the time of oval extrusion. The lymphatics are most numerous and conspicuous only when the corpus luteum is structurally mature. During pregnancy, the false capsule of the early corpus luteum contains very prominent, enlarged lymphatic vessels in addition to the numerous dilated blood vessels (Gillman and Stein, '41).

This proliferation of the lymphatic vessels in the theca surrounding the growing and mature follicle and in the substance of the corpus luteum suggests an active participation of the lymphatics in the physiological activity of the ovary. The vascular invasion which precedes the growth of lymph vessels is probably a response to a new growth, attending as it does to the metabolic needs of such structures as maturing follicles and corpora lutea. The general view is that the hormones from these structures are conveyed to receptor tissues by blood vessels. It seems probable that the primary effect of the hormones is exerted locally and in consequence the growth of adequate channels for transport of further secretions becomes indispensable. While the rapid metamorphosis of follicular granulosa into granulosa lutein cells is taking place, lymphatic channels which will transport the further secretions of these cells, are being developed. In the words of Drinker and Yoffey ('41) "The profusion of lymphatics is believed to be an expression of the frequent need to rid the tissues of protein-containing edema fluid, which appears in the active phase of the sexual cycle". It appears that this statement is directly applicable to the active ovary since the steroid metabolites of the granulosa cells undoubtedly require as great an avenue of channels for their removal and dissemination as do protein-containing edema fluids.

When retrogressive changes commence in the non-pregnant corpus luteum fatty degeneration and fibrosis of the luteum cells takes place. Their usefulness and ability to secrete steroid complexes probably ceases and with this cessation of function the lymphatic vessels begin to disappear. Lymphatic vessels in the regressing corpus luteum are the first structures to vanish. Little information is available regarding the time at which the lymphatic vessels regress in the corpus luteum of pregnancy. In the human female, the corpus luteum may be removed about the sixtieth or eightieth day without interruption of the pregnancy and in the guinea pig, pregnancy is not necessarily terminated by the removal of the corpus luteum as early as 72 hours following copulation (Loeb, '23). It is possible then that the lymphatics of the corpus luteum of pregnancy regress at about this time.

The cyclical development of lymphatic vessels must obviously have some important bearing upon the activity of the ovary and can scarcely be regarded as being unrelated to the endocrine mechanism. The variations which occur in the rate of flow of lymph from the ovary during the cycle must in turn be dependent upon the anatomical variations which occur in lymphatic growth. In those ovaries of non-pregnant animals in which no lymphatic flow could be shown it is conceivable that the injection was performed during a phase early in or preceding follicular development when lymphatic development is minimal. Since the lymphatic proliferation and the formation of a capillary network around the follicle occurs only with maturation of the follicle, one would expect the lymph flow from the ovary to be greatest at this time.

Le Gros Clark states that the proliferation of lymph vessels is an adaptive response to cope with accumulation in the tissue spaces of fluid and metabolites resulting from increased cellular activity and hyperemia. It follows then that when these fluids and hormones of the ovary are no longer present, the need for a lymphatic circulation ceases. When this takes place the rate of lymphatic flow through the ovary decreases.

No less significant in altering the rate of lymphatic flow in the ovary is the venous pressure. It is nevertheless difficult to estimate the relative importance of the changes which occur in the venules and arterioles of the retrogressing corpora lutea and follicles in the control of the venous pressure. It is generally accepted that lymphatic flow is regulated by venous pressure, permeability of capillary walls, movement and functional activity. Drinker et al. ('33) show that an increase in venous pressure causes a prompt increase in lymph flow of the extremities in the unanesthetized dog particularly if accompanied by active motion. It was shown that very slight massage or movement of the ovary was sufficient to produce a rapid flow within the lymphatic vessels leaving the ovary. It is likely that the existence of arterio-venous anastomoses within the ovary may have an important bearing upon the pressures and flow within the lymphatic vessels. Their existence within the substance of the thyroid gland has been shown and there is little doubt that their function here is definitely connected with the fluctuating activity of various groups of thyroid follicles (Modell, '33). The effect of such a mechanism, whereby blood is shunted directly from arteriole to venule without necessarily passing through a capillary bed, in controlling the supply of blood and indirectly the flow of lymph, can hardly be overestimated. In this laboratory we suspect an anatomical connection between lymphatics and arterio-venous anastomoses within the substance of the ovary adjacent to the hilum, similar to those described for skin, the gastro-intestinal tract and thyroid (Le Gros Clark, '39). If such be the case then a physiological control of lymphatic circulation by means of arterio-venous pressure would exist within the ovary itself.

The differences in the rate of lymph flow from the ovary of the late pregnancy guinea pig as compared with that of the non-pregnant or early pregnant animal are very marked. The need for a functioning ovary during pregnancy diminishes as the pregnancy advances, that is, as the placenta develops. The extent to which the growing placenta assumes the functions of the ovary as a source of endocrines varies from species to species. In the guinea pig this function occurs early in pregnancy and the ovary no longer can be regarded as a structure essential for the continuation of the pregnancy. When this supervention of the placenta takes place, the corpus luteum and whatever follicles may have matured during pregnancy, begin to regress. The lymphatic vessels which were intimately related to these follicles and to the corpus luteum now disappear. This lymphatic change is the first histological evidence of regression in the corpus luteum. As pregnancy advances the demands for ovarian secretions is lessened. With this diminution of hormones the lymphatic circulation is retarded as a direct result of a decrease in the number of lymphatic capillaries surrounding the corpus luteum and follicles and a lessening in the amount of available steroid. The slow rate of lymphatic flow encountered in the ovary of late pregnancy can therefore be attributed to the histological changes occurring in the lymphatic vessels which in turn are related to the hormone producing tissues within the ovary.

SUMMARY AND CONCLUSIONS

1. The ovaries of twenty-nine pregnant and forty-five non-pregnant guinea pigs were injected with India ink and the rate of lymphatic flow from the ovaries was determined.
2. The rate of flow varied according to the duration of pregnancy, number of follicles and/or corpora lutea present within the ovary.
3. These variations in the rate of flow were correlated with the specific anatomical changes which are known to occur in the related graaffian follicles and corpora lutea.
4. It was suggested that the lymphatic flow from the ovary of the guinea pig is related to the physiological state of the ovary and may play some part in the transportation of steroids from the ovary.

The author expresses his sincere thanks to Dr. J. Gillman at whose suggestion this study was undertaken, and to Dr. A. G. Oettle, for invaluable criticism and to Prof. R. A. Dart, in whose department this investigation was conducted.

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ON THE EXPRESSION OF POLYDACTYLISM IN THE WINGS OF FOWL

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TWO FIGURES

Polydactylism is a well-known and ancient mutation of fowl. In its most typical manifestation it accounts for the presence of five instead of four toes on each foot and as such is a standard trait of several breeds of chickens.

Polydactylism of fowl is produced by duplication of the anlage material for the first digit. The type and degree of this duplication are very variable and the many resulting forms of expression have been the subject of repeated morphological description. All observations on the genetic behavior of polydactylism in chickens have been based on the structure of the toes.

It was shown, however, by Barfurth ('10, '11, '14) that the wings are also involved in the expression of polydactylism. He found in embryos of polydactylous breeds (Houdan, Orpington, Silky) that on the eighth day of development a small bud-like structure appeared on the radial margin of the hand. This protrusion increased in size during the ninth and tenth day, at the same time acquiring a somewhat more finger-like independence. The formation of pre-cartilage and of small amounts of cartilage were observed, but this did in no case progress to the appearance of phalanges. On the tenth or eleventh day the finger-bud became constricted at its base and this constriction continued until finally the primordium was lost. A similar account of the formation and subsequent loss of such a transitory finger primordium was given by Schmalhausen ('34) for embryos of Faverolle fowl.

We had occasion to make extensive observations on wings of embryos and chicks from polydactylous stock of Houdan-crossbred and Dorking origin. The following results seemed worthy of record.

APPEARANCE AND PERSISTENCE OF SUPERNUMERARY DIGITS

It was possible in our material to distinguish as early as the beginning of the eighth day of development between the presence and absence of polydactylism of the wings. In some of the embryos development of the supernumerary finger buds proceeded in a manner very similar to that described by Barfurth. The histological differentiation of these structures did generally not go beyond the formation of prechondral cells; subsequently the finger primordia became pinched off and were lost. In other instances a slight bulging appeared at the site of polydactylous digit development, but did not progress far in either size or structural differentiation and later was incorporated into general growth of the wing. In yet other cases, however, the formation of permanent appendages of varying sizes occurred.

TABLE 1

Distribution of embryos with signs of wing polydactylism at the ages of 8 to 17½ days, according to whether the parents were homozygous or heterozygous for polydactylism.

AGE IN DAYS	HOMOZYGOUS PARENTS		HETEROZYGOUS PARENTS		TOTALS FOR AGE PERIODS INDICATED			
					Homozygous parents		Heterozygous parents	
	Total examined	Percent wing polydactylism	Total examined	Percent wing polydactylism	Total examined	Percent wing polydactylism	Total examined	Percent wing polydactylism
8	20	100	23	73.9	46	91.3	65	64.6
8½	17	88.2	20	75.0				
9	9	77.8	22	45.5				
9½	305	69.5	356	44.7	305	69.5	356	44.7
10	16	81.3	22	31.8	93	58.1	130	25.4
10½	15	86.7	22	31.8				
11	18	72.2	32	37.5				
11½	14	57.1	17	11.8				
12	11	45.5	21	23.8				
12½	19	10.5	16	0				
13½	15	6.7	6	16.7	77	23.4	58	8.6
14½	15	6.7	9	11.1				
15½	16	25.0	15	0				
16½	14	50.0	12	16.7				
17½	17	17.6	16	6.3				

A summary of the data collected during 1941 is given in table 1. The large number of embryos in the 9½-day group is explained by the fact that most of our observations were made at this particular age. Progenies from inter se matings of homozygous and heterozygous parents are tabulated separately. In both groups there is a progressive decrease, from the eighth day to later stages, in the percentage of embryos with wing polydactylism. The daily samples of embryos are not large enough to give a smooth distribution, but if the data are summarized into larger age groups, it can be seen that among the homozygous embryos incidence of wing polydactylism falls from 91.3% for the 8- to 9-day stage to 23.4% in the 13½- to 17½-day group. Similarly, among the progenies from heterozygous parents the frequency of wing polydactylism is 64.6% in the 8- to 9-day age class, but only 8.6% in that for the 13½- to 17½-day embryos. In homozygous progenies actually all the embryos start out to develop signs of wing polydactylism (eighth day). In progenies of heterozygous parents the incidence of wing polydactylism is lower throughout than it is in embryos from homozygous matings and the frequency of loss or disappearance of finger buds, the formation of which had been initiated, is greater.

The fact that in the older age groups some of the embryos had relatively large finger-like appendages with well-differentiated cartilage suggested that some of these structures would probably have persisted in the hatched and growing chicks. Additional observations on newly-hatched chicks were, therefore, made in 1942. The material came from four different families in which selection had been carried on during several years for particular types of expression of polydactylism. These families differ from each other in two principal features. In one of the four families (pen 7) all animals were homozygous for the main gene for polydactylism, whereas the other three families consisted of heterozygous birds. The homozygous family was selected from that 1941 stock which had given the highest incidence of wing polydactylism. All of the families differed from each other in regard to the presence of particular modifiers which affect the expression of polydactylism.

The highest quantitative degree of expression of reduplication (presence of more than five toes each foot) occurs only in birds which are homozygous for the main genetic factor for polydactylism. Similarly, in the wings, polydactylism occurs more commonly in homozygous than in heterozygous families (table 1; table 2, pen 7), and the size of supernumerary fingers tends also to be greater.

Among the remaining three families two had been selected for heterodactylism, that is, the presence of either four toes on the left foot and five on the right (pen 8), or the reverse (pen 16). Warren ('44) has shown that heterodactylism occurs only in animals which are heterozygous for polydactylism. Our own observations confirm this conclusion. The third family (pen 27) was composed also of heterozygous animals, but here selection had been for the presence of five toes on each foot.

We know that numerous modifying factors exist which prevent maximum expression of polydactylism. With regard to the three families of heterozygous polydactylous birds the more effective concentration of modifiers obtains in the heterodactylous groups (pens 8 and 16). Our data show that these two families of heterodactylous fowl have progenies with a much lower percentage of wing polydactylism than has the progeny of the homodactylous family (pen 27). It seems likely, therefore, that at least some of the modifiers of polydactylism operate simultaneously on the wings and on the feet.

In those chicks which have polydactylous wings at hatching time the supernumerary fingers persist throughout life. Position and size of the extra fingers are very variable. The appearance of one well-developed supernumerary finger in both wings of an adult animal is illustrated in figure 1. A higher degree of wing polydactylism is shown in figure 2. Size of the supernumerary fingers and frequency, or persistence, of their occurrence have been increased by selection. The much slower response of the wings, as compared with the toes, to selection for a high degree of expression (and, in the case of the wings, of persistence) of polydactylism suggests that there are some modifiers (suppressors) which affect only the wings.

TABLE 2

Frequency of wing polydactylism among newly hatched chicks of four different families.

PEN	TYPE OF MATING	NUMBER OF CHICKS EXAMINED	PERCENTAGE OF WING POLYDACTYLISM
8	Heterozygous polydactyl inter se (4L/5R)	87	9.2
16	Heterozygous polydactyl inter se (5L/4R)	188	4.8
27	Heterozygous polydactyl inter se (5L/5R)	396	27.0
7	Homozygous polydactyl inter se (6L/6R)	69	59.4

ASYMMETRICAL DEVELOPMENT OF WING POLYDACTYLISM

Among heterodactylous fowl animals with five toes on the left foot and four on the right are much more common than those with four toes on the left foot and five on the right (Bond, '20, '25–'26; Fisher, '35; Vainikainen, '35; Wodzicki, '36; Warren, '44). Only one exception to this rule is on record (Punnett and Pease, '29). The same preponderance of the left side in the expression of polydactylism holds for birds which have five toes on one foot, but six on the other. Moreover, even

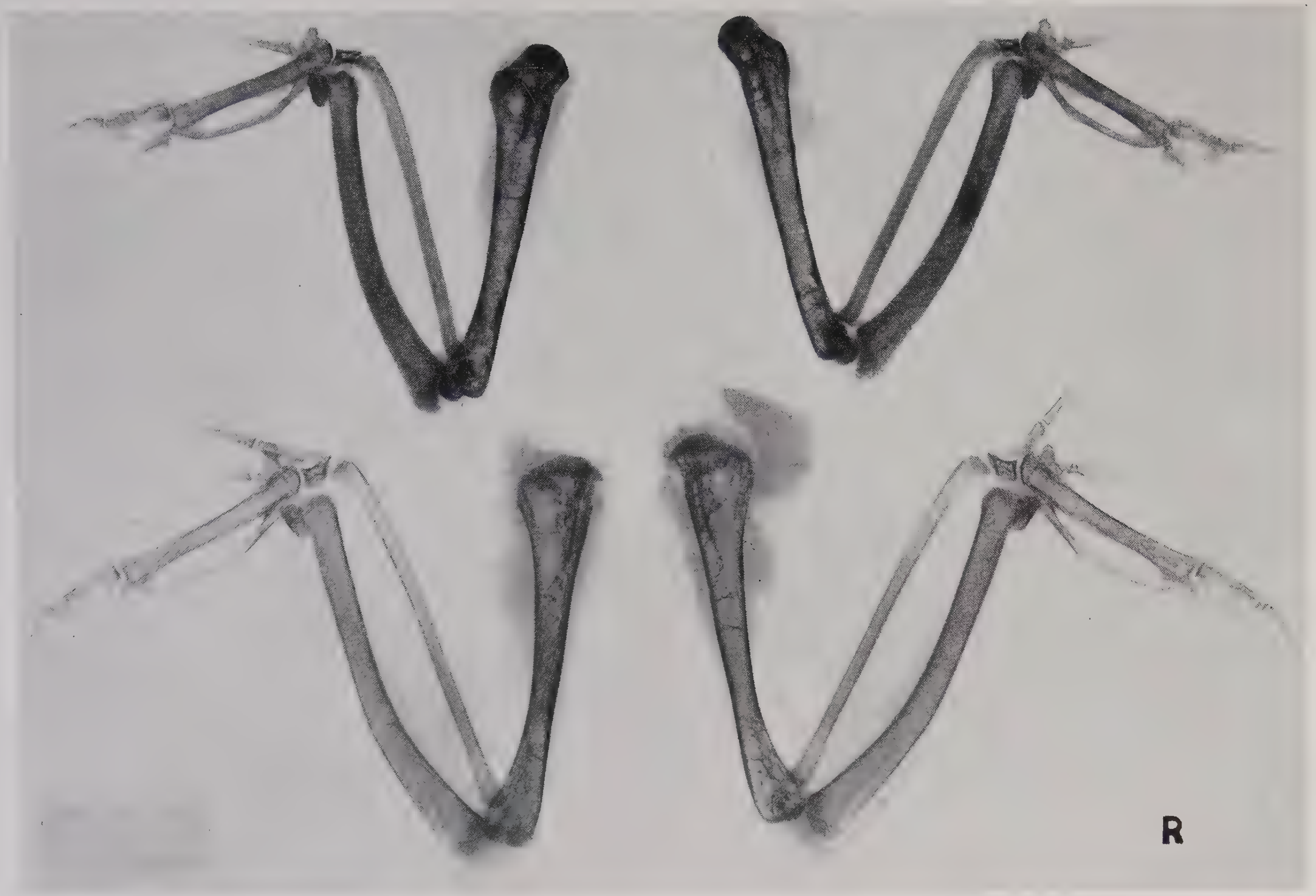

Fig. 1 Wings of a normal Leghorn fowl, above and a polydactylous one (♂5794, age 4 months), below. In ♂5794, both wings bear a supernumerary finger, similar in size and structure to the normal second digit.

among those homodactylous animals which show lateral size differences of the supernumerary toes, the toes on the left foot tend to be the larger ones.

It seemed of interest to determine whether or not similar relationships exist with regard to the expression of polydactylism in the wings. In 1941 we made observations with this in mind on a large group of embryos from several matings of polydactylous fowl. The data are assembled in table 3. The embryos are grouped according to the polydactylous condition of the toes. The group designated as "4½–4½" comprises embryos with poor development of the supernumerary toe; the "6–6" groups includes all embryos which had more than five toes on either foot.

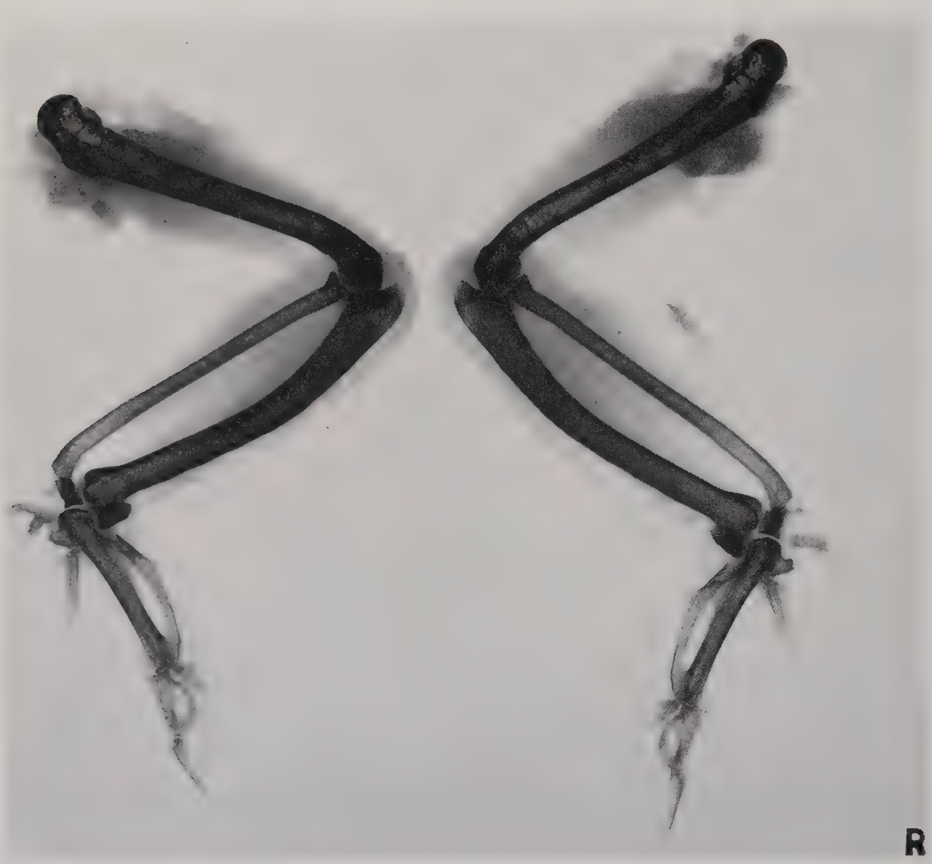

Fig. 2 Wings of ♂6529, age about 6 months. More extensive wing polydactylism than that illustrated in previous figure.

It can be seen at once that in all groups, except the one with four toes on the left and five on the right foot, the left wing, as compared with the right one, shows by far the greater amount of supernumerary digit development. The reverse, viz., predominance of polydactylous development in the right wing, is found among those embryos which have five toes on the right foot but only four on the left.

Similar observations were made in 1942. Among thirty-three embryos with a 6–4, 5–4 or 6–5 toe condition [1] seven had supernumerary digits on the left but not on the right wing and none showed the reverse situation. Among thirteen embryos with 4–5 or 5–6 toes there were three with polydactylism of only the right wing and none of the opposite type. Among 115 newly hatched chicks with 4½–4, 5–4, 6–4, or 6–5 toes there were fourteen with unilateral polydactylism of the left wing and only one which was polydactylous on the right but not on the left wing. Among thirty-three newly hatched chicks with 4–5 or 5–6 toes three had unilateral right wing polydactylism and only one was of the left type. This 1942 material includes 194 embryos and chicks with heterodactylous feet and in 76.3% the excess was found on the left foot. There were thirty embryos and chicks with heterodactylous wings and in 76.7% of them polydactylism existed only on the left wing.

TABLE 3

Occurrence of polydactylism on right and left side of feet and wings.

NUMBER OF TOES ON LEFT AND RIGHT FOOT (LEFT GIVEN FIRST)	NUMBER OF EMBRYOS	OCCURRENCE OF WING POLYDACTYLISM IN PERCENT	PERCENTAGES OF DIFFERENT TYPES OF WING POLYDACTYLISM AMONG ALL WITH POLYDACTYLOUS WINGS		
			Similar degree of expression both wings	On left wing only or definitely more so than on right	On right wing only or definitely more so than on left
5 –4	73	23.3	47.1	41.2	11.8
4 –5	27	40.7	54.6	18.2	27.3
4½–4½	94	29.7	46.4	46.4	7.1
5 –5	256	47.6	40.1	48.3	11.5
6 –6	187	69.4	53.9	36.2	10.0

As in the case of our data concerning the frequency of wing polydactylism in different families, the observations on asymmetrical occurrence of supernumerary fingers point definitely to the conclusion that some of the factors which operate as modifiers of the toe expression of polydactylism do so also in the wings. In neither of the two cases can selective mortality have played a causative role.

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[1] Number of toes on left foot always given first.

We have no definite information concerning the nature of the factors which lead to asymmetrical expression of polydactylism in feet and wings. It has been shown that in ordinary, i.e., non-polydactylous, chickens fresh weight of the long bones of both extremities tends to be greater on the left side than on the right (Weakley and Dustman, '39). It has also been shown that transplants from the left body side of chicken embryos which are in the head-process stage have greater developmental potentialities than similar transplants from the right side (Rawles, '36). On the other hand, it has been found that the growth-restricting conditions of heterotopic limb transplantation (wings and legs) in chick embryos are relatively frequently associated with hypodactyly and hypophalangy (Hamburger and Waugh, '40). It seems reasonable to assume that agencies which promote increase in general size of the skeletal parts of the extremities on one body side or which stimulate preferentially one whole body side of the embryo also act as modifiers of the gene for polydactylism.

SUMMARY

Observations on the wings of embryos, chicks and adult fowl of polydactylous stock gave the following results.

In confirmation of observations by Barfurth and Schmalhausen it was found that supernumerary finger primordia may be formed in embryos of polydactylous fowl and may subsequently be lost. Such loss of initially-formed finger buds is more common among embryos from parents which are heterozygous for polydactylism than in homozygous progenies.

In other cases, however, the supernumerary primordia persist and develop into well-differentiated digits. Persistence of the expression of wing polydactylism is more common in animals which are homozygous for polydactylism than it is in heterozygous ones.

As in the case of the toes heterodactylism in the wings is more frequent on the left side than on the right. Animals which have heterodactylism of the right foot, tend also to have heterodactylism of the right wing.

The modifying factors which influence the degree of expression of polydactylism of feet and wings appear to be in part identical, in part different ones.

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VACUOLATION OF THE ACINAR CELLS IN THE PANCREAS OF THE RAT AFTER TREATMENT WITH THYROXINE OR ACETYLCHOLINE

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FIVE FIGURES

During a systematic examination of the organs of rats treated with thyroxine, it was found that vacuoles containing various cellular elements were frequently present in the acinar cells of the pancreas.

Perusal of the literature on the subject revealed that only Kojima ('17) had reported the presence of vacuoles in pancreatic acini after thyroid treatment; however, these vacuoles were described as being empty. It was therefore, considered worthwhile to carry out a methodical study on the effect of thyroxine on the histology of the pancreas using modern staining techniques.

Vacuolation of the acinar cells in the pancreas has also been induced by completely different methods, such as treatment with pilocarpine in the dog (Mouret, 1895) or electric stimulation of the vagus nerve in the dog (Babkin and coworkers, '09). Since both methods implied that an increase in parasympathetic activity (or vagal tonus) could determine the formation of vacuoles, it was decided to test the most active parasympathetic drug, acetylcholine, in the rat. A pancreatic vacuolation identical with that resulting from the injections of thyroxine was obtained.

Experiments were then carried out to decide which of the two factors — thyroid hormone or parasympathetic tonus — was the primary cause of the vacuolation of the acini in the pancreas. A partial answer to this question, as well as a description of the vacuoles, will be found below.

EXPERIMENTAL

Thirty-six female rats weighing from 70 to 90 gm. were divided into six groups of six animals. Two groups were immediately thyroidectomized. Five days later, two other groups were treated with an injection of 0.3 mg. of thyroxine. This dose, which is known to produce a marked hyperthyroidism (Leblond and Hoff, '44) was repeated daily. Autopsy was performed 12 days after the beginning of the experiment, that is to say 6 days after the start of the thyroxine treatment. Previous to autopsy all animals were fasted overnight; and one group of each type (normal, thyroidectomized and thyroxine treated) was given three injections of 1 mg. of acetylcholine at 1 hour intervals; the animals being sacrificed 1 hour after the last injection. In the injected solution enough eserine was added to have 0.08 mg. of this substance in each dose of acetylcholine and thus prolong the effect of the latter.

The pancreases were taken immediately after death, preserved in trichloracetic Bouin and stained according to Gomori's method. In addition, Masson's trichrome, Sudan III and routine hematoxylin — eosin were used in a few cases.

EFFECTS OF THYROIDECTOMY AND THYROXINE TREATMENT

In table 1 it will be seen that thyroidectomy decreases the weight of the pancreas, as reported by Hammett ('26) while thyroxine increases the weight of this organ in confirmation of results obtained with thyroid extract by Herring ('17) and Cameron ('26).

TABLE 1

	AVERAGE BODY WEIGHT (GM.)		AVERAGE PANCREAS WEIGHT (MG.)	VACUOLATION OF ACINAR CELLS
	Beginning	Autopsy		
Normal	82	92	305 (270–335)	0
Thyroidectomized	75	74	231 (165–270)	0
Thyroxine	88	91	413 (340–460)	+ +
Normal + acetylcholine	80	98	295 (275–380)	+ + +
Thyroidectomized + acetylcholine	79	83	260 (215–301)	+
Thyroxine + acetylcholine	87	76	397 (301–465)	+ + + +

The extreme variations in pancreas weight are recorded in parentheses following the average weights of the pancreas for each group.

Microscopic examination, however, does not show distinct differences in the cell sizes of the various groups as might be expected from the weight differences. There were, however, clear-cut variations in the amount of zymogen granules present in the cells. Since the animals had been fasted, the pancreatic cells were loaded with zymogen granules, at least in the normal controls. The amount of granules in thyroidectomized animals was definitely smaller than in normal rats.

Among the thyroxine treated animals, three showed a granular surcharge at least as marked as in normal controls; but two of the hyperthyroid rats had acini containing only a small proportion of granules. The two hyperthyroid rats with very few zymogen granules had as heavy pancreases as the others. It seems therefore that the amount of granules can play only a minor rôle, if any in causing the weight differences between the pancreases in the various groups.

On the other hand mitoses were completely absent after thyroid removal; they were rare in normal controls, and very common in rats given thyroxine. Therefore, it appears that the weight of the pancreases varies with the number of mitoses, suggesting that the thyroid hormone acts on the pancreas through an hyperplasia of the cells rather than through an individual hypertrophy. The opposite is true in the action of thyroxine on the heart and kidney (Leblond, '44).

In all the animals treated with thyroxine there were vacuoles in the cytoplasm of some of the acinar cells (fig. 4). This was not necessarily the case when thyroxine was given under different conditions, since in experiments carried out with the same dose of thyroxine for a longer time (21 days) only three out of six animals showed a similar vacuolation.

The vacuoles were usually present in all cells of contiguous acini, forming groups which could be easily found under the low power of the microscope (fig. 4). Sometimes, however, a single cell was vacuolated. There were in all cases fairly large regions in which the acinar cells appeared normal.

The vacuoles were either empty, or loaded with various cellular materials. The vacuoles which appeared empty were frequently very small and located among the zymogen granules. When they were present in other parts of the cells, they were much larger. The largest one occupied the whole cell flattening the nucleus; figures similar to those of fatty degeneration were thus obtained. It must be noted, however, that repeated attempts at detecting fat with Sudan III in formalin-fixed pancreases showed the absence of fatty substances in all types of vacuoles described in this work.

In the case of the vacuoles containing cellular elements, the material most commonly found in their interior was clumps of zymogenic granules. These were either distinct or more or less agglutinated. Sometimes a solid mass of red colloid, possibly resulting from the fusion of the granules, would fill a vacuole. Frequently masses of cytoplasm looking like the basophilic part of the acinar cells were identified in the vacuoles. The nucleus itself, with or without accompanying cytoplasm, could be enclosed in the vacuolar formations. These intra-vacuolar nuclei appeared either normal or pycnotic. Finally, numerous vacuoles contained fuzzy looking formations which could not be linked with parts of the cells (fig. 3).

It must be noted that a few rare cells with vacuoles were encountered in two of the six normal controls; one vacuolated cell was seen in one of the six thyroidectomized animals.

In a large group of older rats (200 to 300 gm.) we have seen several cases of animals with vacuoles in the pancreatic acini, although no treatment had been given.

EFFECT OF ACETYLCHOLINE

The acute treatment with acetylcholine in normal rats produced a considerable discharge of zymogen granules as previously reported (Sergeyeva, '38, '40). In addition, a marked vacuolation was found in the cells of the pancreatic acini. The vacuolation was more extensive than after thyroxine treatment; although the structure of the vacuoles was identical in both cases, showing the two types of formations — the empty vacuoles and those containing cellular material (fig. 5).

Vacuoles were present in the six normal rats treated with acetylcholine. Experiments carried out in similar conditions in rats of various sizes showed a vacuolation of the pancreas in every single instance (fig. 2). In a few animals sacrificed as early as 30 minutes after the first injection of acetylcholine, the only vacuoles to be seen were small, numerous, empty cavities present among the zymogen granules which had not yet been discharged.

Even the thyroidectomized rats, which appeared less affected by the dose of acetylcholine than the normal controls as far as tears and diarrhea were concerned, responded with vacuolation of the acinar cells of the pancreas. However, acetylcholine produced less widespread vacuolation in their case as in the normal animals. On the other hand the

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Fig. 1 Normal pancreatic acini — (Hematoxylin — eosin. High magnification).

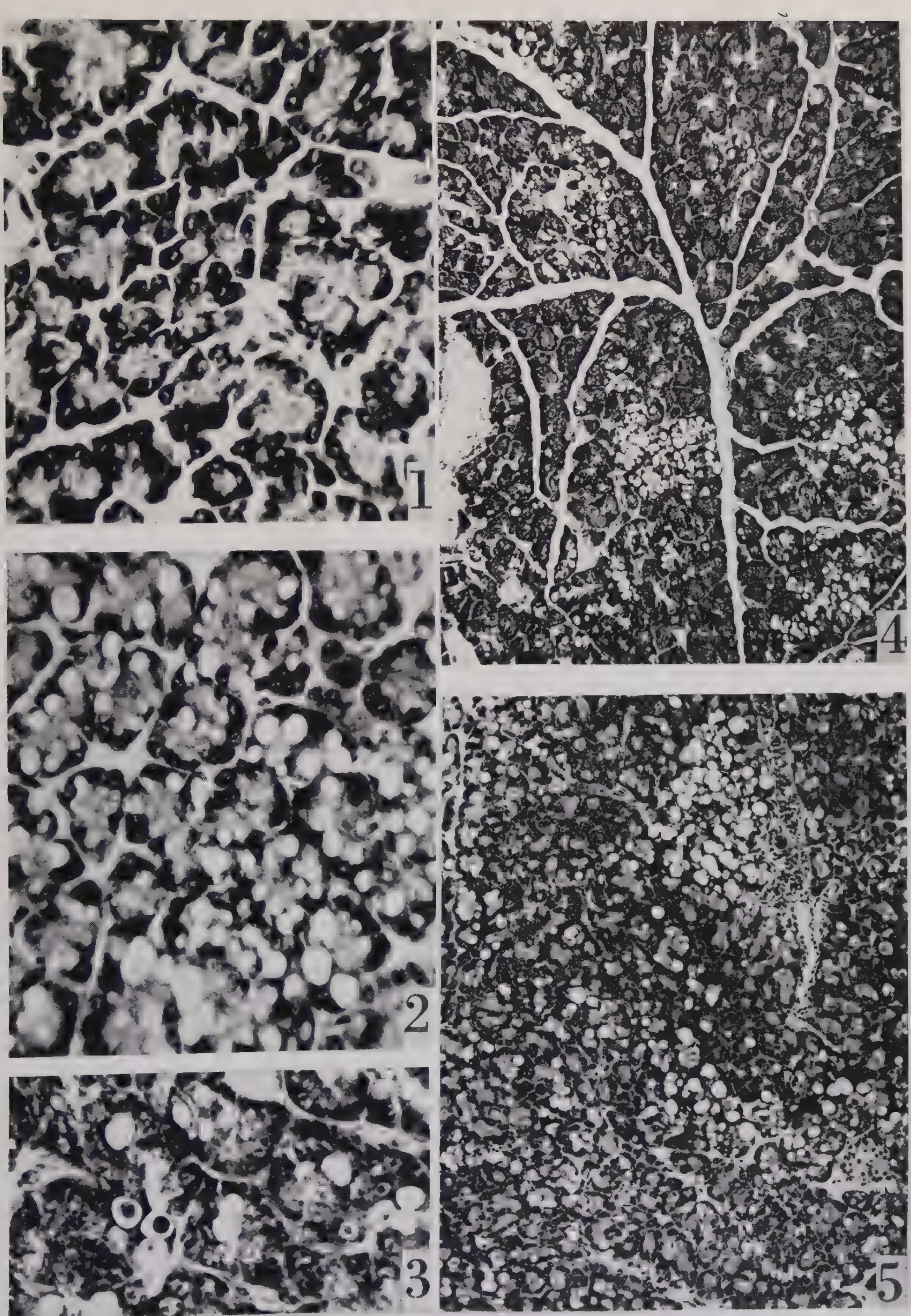
Fig. 2 Pancreatic acini with vacuoles due to acetylcholine treatment. (Hematoxylin — eosin. High magnification).

Fig. 3 Pancreatic acini with vacuoles, some empty and some containing cellular material, after thyroxine treatment (Gomori stain. High magnification).

Fig. 4 View of the pancreas at a low magnification after thyroxine treatment (Gomori stain). Several clumps of vacuolated cells may be seen.

Fig. 5 View of the pancreas at a low magnification after acetylcholine treatment (Gomori stain).

In non-vacuolated areas it is possible to see that the centers of the acini are light owing to the discharge of zymogen granules. In vacuolated areas cellular material may be encountered in some vacuoles.



Figures 1 to 5

thyroxine treated animals which received acetylcholine appeared extremely sensitive to this drug, since only two of the six survived the three injections. The vacuolation was intense in these animals, suggesting a cumulative effect of acetylcholine and thyroxine; which was visible even in those animals that died after one or two injections. In the two survivors, there were extreme phenomena of vacuolation extending, to every single cell of the pancreas, with many cells undergoing a complete degeneration.

EFFECT OF DINITROPHENOL

Since one of the main effects of the thyroid hormone is to activate the metabolism, the effect of a metabolic stimulator, dinitrophenol, which does not affect thyroid deficiency (Leblond and Hoff, '44; Leblond, '44) was examined. Six female rats of 200 ± 20 gm. were given subcutaneously 30 mg. of dinitrophenol per kilogram of body weight and sacrificed at the peak of the metabolic stimulation 30 minutes later. No vacuolation of the acinar cells was present in their pancreases.

DISCUSSION

Since the vacuoles first appeared among the zymogen granules, it is logical to assume that they result from an activation of the trypsin presumably connected with these granules. Indeed, Villaret and coworkers ('29) found that the pancreatic secretion produced by acetylcholine in the dog could digest proteins without addition of enterokinase; they concluded that an activated trypsine had been secreted in these conditions. Therefore, the vacuoles may be explained as an autodigestion of the cell which originates in the group of zymogen granules and may extend to the rest of the cytoplasm, including the nucleus at times and leading to extensive degenerations. It is interesting to note that Villaret and coworkers ('29) believed they had produced an acute pancreatitis by injections of acetylcholine to the dog.

It is clear that acetylcholine does not produce these degenerative changes in the pancreas through some action on the thyroid gland, since the drug is effective in thyroidectomized animals. The vacuoles observed appear to be due to the direct influence of an increased parasympathetic tonus, producing along with the discharge of the zymogenic granules, an activation of some of them in situ. The effect of acetylcholine being minimal in thyroidectomized animals and maximal after thyroxine treatment, it is apparent that the response of the pancreas to parasympathetic stimuli is greatly facilitated by thyroxine; so much so that in case of thyroxine overdosage without addition of acetylcholine, the normal parasympathetic impulses are sufficiently potentiated to produce a vacuolation of the pancreas. The alternative explanation, that thyroxine is directly vagotonic, although it would act more slowly than acetylcholine, is not in accord with known facts (for instance, thyroxine accelerates the heart, etc.).

SUMMARY

Overdosage with thyroxine for 6 days produces a characteristic vacuolation in some of the acinar cells of the pancreas (fig. 4), similar to that obtained with an acute treatment by acetylcholine (fig. 5).

These vacuoles may appear empty or contain various types of cellular fragments, even the nucleus. They are interpreted as resulting from an intra-cellular auto-digestion due to an activation of trypsine which originates among the zymogen granules and extends to the rest of the cell.

Since the effect of acetylcholine is still present after thyroidectomy, this drug acts on the pancreas directly and not through the thyroid gland. Thyroidectomy, however, reduces and thyroxine increases the ability of acetylcholine to produce vacuolation of the pancreas. Thyroxine appears to potentiate acetylcholine as far as its effect on the pancreas is concerned. The possibility of obtaining vacuoles with thyroxine alone is interpreted as a potentiation of the normal parasympathetic impulses by this hormone.

Incidentally it was noted, that the growth promoting effect of thyroxine on the pancreas is the result of hyperplasia of the acinar cells without apparent hypertrophy.

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A TECHNIQUE FOR THE ARTIFICIAL INSEMINATION OF MICE

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TWO FIGURES

Long and Mark ('11), in their classical study of the maturation of the egg of the mouse, used artificial insemination in order to obtain the eggs of a known post-insemination age. The time of birth of litters was determined by a special mechanical contrivance. Insemination was performed at from 9½ to 28½ hours after parturition. Spermatozoa from the vasa defferentia were suspended in tepid physiological saline (0.75%) and drawn into a fine glass syringe. The mouse was held in the left hand between two pieces of cotton batting. A glass speculum introduced into the vagina made it possible "to see the somewhat constricted orifice of the neck of the uterus, and to introduce into the uterus by means of the syringe operated by the right hand a very few drops of the fluid containing spermatozoa." It was found desirable to keep the speculum and syringe at body temperature. The females were killed at varying intervals following insemination, up to 40 hours post partum. In all, 149 mice were inseminated, but of these only eighty-five were sufficiently studied to determine whether or not fertilization had occurred. Of the eighty-five, fourteen had not ovulated at the time they were killed and hence can be disregarded. Of the remaining seventy-one, thirty-one or 43.7%, furnished eggs some of which contained spermatozoa or pronuclei.

Some of the failures were probably due to the fact a number of the inseminations were performed too soon after parturition. All but two of the thirty-one fertile inseminations were performed between 16½ and 28½ hours post partum while most of the infertile inseminations were performed 11 to 18½ hours post partum. Ovulation, according to the data of Long and Mark, occurs between 14½ and 28½ hours post partum, thus nearly coinciding with the optimum period for insemination which may be taken as the interval 18½ to 28½ hours after parturition. The time of day or night at which the inseminations were performed is not stated, but since the modal hour for births falls at about

2 or 3 A.M. (Long and Mark, '11; Merton, '38), the optimum hour for most inseminations would come during the night.

No young were carried to term, but it is stated that three young were obtained from a female rat similarly inseminated. In view of the fact that in the mouse and rat, embryos do not implant unless the mother has copulated or been subjected to some other stimulus adequate to induce the progestational condition of the uterus, there is some reason to doubt if many of the fertilized eggs would have survived. The expectation in this case depends on the somewhat uncertain probability that the stimulation of the cervix involved in the introduction of sperm into the uterus would be adequate to induce the progestational condition.

Merton ('39) also artificially inseminated mice at the first post partum estrus. The technique is not described in detail, but was evidently closely similar to that used by Long and Mark. Four animals are listed as yielding fertilized eggs.

Blandau and Jordan ('41) have described a technique for the artificial insemination of the rat. Estrus was determined by the copulatory response. When heat was indicated, the cervix uteri was stimulated by a strong faradic current. The female was then lightly anesthetized with ether. An ear speculum was placed deep in the vagina and, with the aid of light from a head mirror, a hypodermic needle inserted in turn into each cervical canal and 0.1 cc. of sperm suspension injected. An artificial vaginal plug was then prepared by dipping a small piece of cotton, first, into the secretion of the seminal vesicle of a killed male, second, into the secretion of the coagulating gland, and immediately transferring it to the vagina through the speculum. Over 500 female rats were successfully inseminated by this technique, the percentage of impregnations being as high as in animals allowed to copulate normally.

Snell, Hummel, and Abelmann ('43) have published a preliminary account of a technique of artificial insemination for mice. The inseminations described in this account are those listed below as series III.

THE TIME OF ONSET OF ESTRUS

Mice usually come into heat sometime during the night. If females showing an estrous smear are placed with males and examined at frequent intervals for vaginal plugs, the first plugs are almost never found before 10 or 11 P.M. In the rat, on the other hand, the onset of estrus usually occurs between 4 and 10 P.M. (Blandau, Bolling and Young, '41). Fortunately for anyone undertaking a project which calls for the use of animals in heat, the onset of estrus has been shown to be determined by the diurnal light cycles (Hemmingsen and Krarup, '37; Browman, '37, for the rat; Snell, Fekete and Hummel, '40, for the mouse) so that by using an artificially controlled alternation of light and dark the modal hour for the onset of estrus can be shifted backwards or forwards to any desired degree. Using this method, Snell, Fekete and Hummel have concluded that the onset of estrus, as determined by the time of appearance of vaginal plugs most commonly occurs between 10 P.M. and 1 A.M., and that ovulation most commonly occurs between 12 P.M. and 2 or 3 A.M.

Since it presumably is important that artificial inseminations be performed when a female is actually in estrus, it was deemed advisable to get still more accurate information about the onset of estrus in the mouse. An attempt was made to do this by using the copulatory response which Young and his co-workers have used so effectively for the rat. However, while some mice seemed to show the response quite definitely we were unable to conclude that it is a reliable indication of heat for this species. Consequently we returned to the method, previously used, of checking for the hour of plug formation.

Three series of animals were run at different times, as follows: 1, B alb C females left in a dark room with lights on at 10:30 P.M., off at 10:30 A.M. total of thirty-three plugs; 2, B alb C females with lights on at 3 P.M., off at 3 A.M. total of nine plugs; 3, Swiss X B alb C hybrid females with lights on at 10:30 P.M., off at 10:30 A.M., total of fifty-eight plugs. The results are shown graphically in figure 1. The triangles indicate the approximate hours at which observations were made. Departures of ½ hour or more from the selected hour for plug-checks occurred on a number of occasions. The data show a definite mode about 4 to 6 hours after the beginning of the dark period. Apparently the onset of estrus is earlier in the hybrid mice, but it is quite possible that this difference is due to seasonal differences or to other factors.

THE NORMAL FERTILITY PERCENT

In order to determine the optimum conditions for fertile inseminations, some observations were made on the fertility of normal matings under various conditions. The fertility will be expressed as the fertility per cent, defined as the number of fertile matings divided by the total number of matings (plugs) times 100.

Under normal lighting conditions, B alb C mice whose last litter was killed at birth showed 84% fertility at the second post partum estrus (38 matings), and 95% fertility at the first post weaning estrus (38 matings). The difference is not significant ($P = .15$) but nevertheless was one of several factors that led us to use the first post weaning estrus as the estrus for artificial inseminations. We have no data for the first post partum estrus, but some information concerning matings at this period has been provided by Crew and Mirskaia ('30). These authors found that fruitful mating at the first post partum estrus "is exceedingly rare in the suckling mother, but if the litter is removed immediately after birth the mating associated with the estrous phase which normally follows within 24 hours after parturition is, with few exceptions, followed by pregnancy of normal duration." Their figures show 258 vaginal plugs occurring in non-sucklung females, followed by 198 pregnancies. It is not entirely clear whether all these plugs occurred at the first post partum estrus, or whether some were second plugs following a first sterile mating. In any case we may conclude that the fertility of matings at the first post partum estrus did not exceed 76.8%.

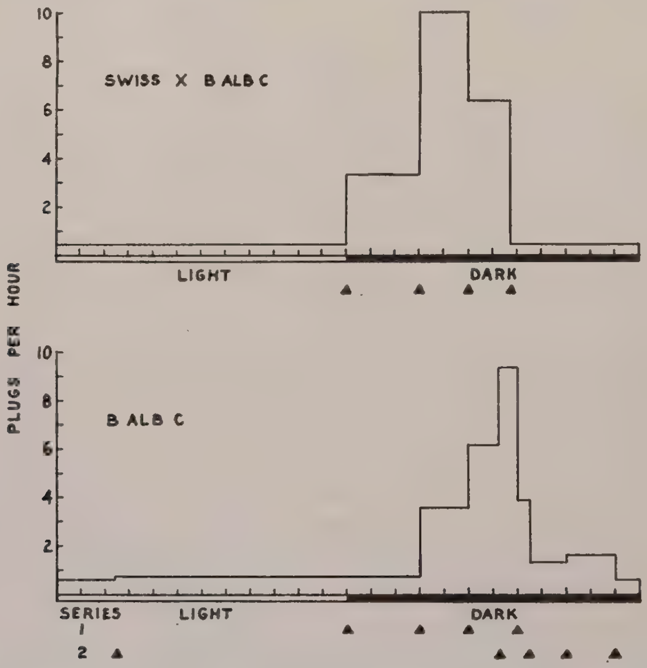

Fig. 1 The relation of the onset of estrus, as determined by plug formation, to the onset of darkness under conditions of artificially controlled light and dark. Triangle indicate the periods in the light cycle when checks were made for plugs.

The above figures are based on observations made under normal lighting conditions. The use of a reversed light cycle introduces additional factors which might affect fertility percent. Some early observations strongly suggested that length of adaptation to the light cycle was one of these. Consequently tests were made bearing on this point. A colony of Swiss X B alb C hybrid females was maintained for 16 days in a darkroom with lights on at 4 P.M., off at 4 A.M. On November 27th the lights were shifted to on at 10 : 30 P.M., off at 10 : 30 A.M. Observations on the fertility percent before and after the changes are summarized in table 1. It will be seen that the fertility percent dropped from 100 (6 matings) for the week prior to the light change to 63.2 for the week following the light change, and then rose to 81.5, 90.9, and 93.7 in the following 3 weeks. Evidently at least 2 weeks of adaptation to a new light cycle are necessary for normal fertility.

TABLE 1

Effect of a sudden shift in the light cycle on the fertility of normal matings.

DATE	11/23 11/26	11/27	11/29 12/3	12/6 12/10	12/13 12/17	12/20 12/25
Number of matings	6	lights	19	27	22	16
Number fertile	6	changed [1]	12	22	20	15
Percent fertile	100%		63.2%	81.5%	90.9%	93.7%

[1] Cycle prior to 11/27: lights on at 4 P.M., off at 4 A.M. Cycle after 11/27: lights on at 10 : 30 P.M., off at 10 : 30 A.M.

TECHNIQUE OF ARTIFICIAL INSEMINATION

Four series of artificial inseminations were run, each of the last three embodying improvements suggested by the series preceeding it. Only the last series will be described in detail.

In series 1, females which had just mated to a vasectomized male were inseminated under nembutal anesthesia by operative procedure, the sperm being introduced directly into the uterus by the technique developed by Fekete and Little ('42) for the transfer of ova from the oviduct of one female to the uterus of another. While we know of no theoretical reason why this method should not give satisfactory results, the fertility percent was in fact disappointingly low, varying from 10% to 44% in the several groups of animals used.

Series II differed from series IV in that the females were inseminated under ether anesthesia, and in several other presumably less important respects. The results were not satisfactory.

Series III differed from series IV principally in that the success of the inseminations was determined by killing the females in the early stages of pregnancy.

For series IV, a colony of 144 Swiss X B alb C hybrid females mated to Swiss males was maintained in a well ventilated darkroom in which the lights were turned on at 10 : 30 P.M., off at 10 : 30 A.M. by an automatic switch (designed for poultry houses). Pregnant females were isolated in separate pens. On Monday mornings, all litters of 21–24 days of age or older were removed from the mothers and vaginal smears taken at once, and again on the following three or four mornings if estrus had not occurred meantime. Inseminations were performed at about 4 P.M. each day on all test females whose smears indicated estrus. Usually an additional group of females with estrous smears was returned to the home pens at 4 P.M. to serve as controls.

Smears were taken with a wire loop, allowed to dry on the slide and stained with Wright's stain. Where leucocytes were absent or nearly absent, a cell count was made with the aid of a piece of cellophane ruled in squares and inserted in the eyepiece of the microscope.

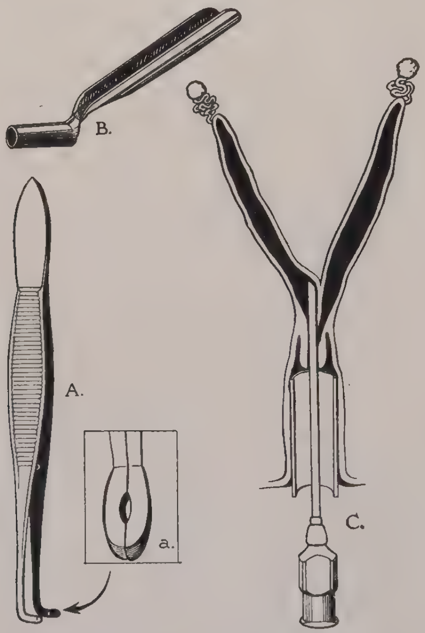

Fig. 2 Instruments used in the technique of artificial insemination: A, forceps for stripping sperm from the vas deferens. B, vaginal speculum. C, blunt pointed needle, inserted into the left horn of the uterus by turning the point to the left.

The inseminations were performed with the aid of an especially designed speculum and a head mirror. The speculum, outside diameter at smaller end 3 mm., at larger end 3.5 mm. (fig. 2 B), was designed with a handle with a grooved upper surface. The female was held by the tail with the left hand, the speculum inserted in the vagina, the tail laid in the grooved handle and clipped in place with a spring-type clothes pin, thus securing the speculum in place. With the aid of light from the head mirror, a 19-gauge needle attached to a tuberculin syringe containing the sperm suspension was then inserted, first into one horn of the uterus and then the other, and about 0.035 cc. of sperm suspension injected into each horn.

In series II a needle was used with the point entirely removed, giving at a square end. With this needle, however, as indicated both by the small size of the litters obtained and tests with dead and partly dissected females, the tip almost invariably entered the same horn of the uterus on successive insertions, no matter what manipulations were used. Consequently in series III a needle with a blunted point was substituted. By turning the point first to the right and then to the left the needle could be made to enter first the right and then the left horns (fig. 2 C).

A clip, made from a safety pin, placed on the plunger of the syringe and adjusted to a new position after the insemination of each horn, served to limit the injection of sperm suspension to any predetermined amount. The clip could also conveniently serve as a pointer to show which way the tip of the needle was facing.

Individual inseminations, made by one operator working alone, required about 4 minutes each including the time necessary for handling the mice and the making of records.

Immediately following each insemination, females were placed with vasectomized males and checked 1 or 2 hours later and again the next morning for plugs.

Sperm for insemination were prepared by stripping from the vasa defferentia of newly killed Swiss mice directly into a vial of medium. The males used had previously been employed for routine matings in the darkroom colony. The process was simplified by the use of stripping forceps (fig. 2 A and a) made by soldering a piece of copper wire to each tip of a pair of straight pointed forceps, bending the wire at right angles, and grinding, on the opposed faces, grooves that would apply just the correct pressure to the vas for effective stripping.

The medium used was: Locke's 37 cc., distilled water 13 cc., dextrose 0.54 gm. The amount employed ranged from 0.25 cc. per two vasa to 1.25 cc. per two vasa. Assuming that two vasa yield 15,000,000 sperm, a figure indicated by numerous counts as a fair average, the sperm per insemination would range from 800,000 to 4,000,000. The quality of the sperm was checked at least once under the microscope; in all later inseminations twice, both before and after the inseminations.

RESULTS

The diagnosis of estrus

Because the vaginal smear does not provide an infallible guide to heat, it was often necessary to inseminate the same female two or more times on successive days. Thus in series IV (table 4), 153 inseminations were performed on 119 females. (In a few cases the same female was used for inseminations following successive litters. Such a female would be counted twice.) The accuracy of the smear as a guide to estrus varied from week to week and apparently increased with the age of the females (or varied with the season?) It is also noteworthy that the older females yielded more profuse estrous smears, as indicated by the cell count, the change being so marked that it can hardly have been due to variations in the method of taking the smear. The females used in series III (table 3) showed a much higher proportion of failure to mate following insemination then was the case in series IV. The explanation is probably to be found in the fact that the darkroom used throughout this experiment was not adequately heated.

The condition of the cervix showed an interesting correlation with estrus. Three facts in regard to the condition of the cervix were recorded at each insemination of series IV except for the first week. (1) The moisture, this being graded as 0, ±, +, or + +. (2) The tension, as determined by the feeling of friction during the entry of the needle, this being graded as 0, ±, +, or + +. (3) Leakage following the removal of the needle. This was either absent or pronounced, hence was graded 0 or +. The results are tabulated in table 2. It will be seen that high tension, absence of moisture, and absence of leakage are indicative of estrus. Of thirty inseminations when the grade was tension + or + +, moisture 0, leakage 0, twenty-seven were followed by plug formation; of thirty inseminations when the grade was leakage +, combined with varying degrees of tension and moisture, only three were followed by plug formation the same evening, seven more the next morning. Intermediate grades showed intermediate degrees of plug formation.

Fertility of the inseminations

Of the 119 females inseminated, ninety-six gave plugs. In the case of thirteen of these, there was leakage at the time of the insemination; these were all sterile. The remaining eighty-three are divided into two groups, those in which the plug was noted at 5–6 P.M. of the day of the insemination and those in which the plug was noted the next morning. The former were 69.0% fertile (58 plugs) the latter 44.0% (25 plugs). The corresponding figures for the control are 91.2% (34 plugs) and 46.2% (13 plugs). The reason for the lower fertility following late plug formation is not apparent. Loss of viability of the sperm might account for this in the case of the test animals, but could not explain the sterile late matings in the control group.

TABLE 2

Relation between condition of cervix and plug formation in mice showing estrous smear.

Tension	+ +, +	+ +, +	+ +, +	±, 0	±, 0	±, 0	0, ±, +
Moisture	0	±	+	0	±	+	±, +, + +
Leakage	0	0	0	0	0	0	+
Plug in P.M.	21	20	9	0	6	3	3
Plug in A.M.	6	5	1	0	3	5	7
No plug	3	9	5	2	5	6	20

TABLE 3

Data relative to fertility of artificial inseminations, series III.

TOTAL NUMBER OF INSEMINATIONS	NUMBER OF FEMALES INSEMINATED	NUMBER OF PLUGS	PLUG OBSERVED <2½ HOURS AFTER INSEMINATION			PLUG OBSERVED >2½ HOURS AFTER INSEMINATION		
			Fertile	Infertile	Percent fertile	Fertile	Infertile	Percent fertile
52	46	19	13	3	81.3%	0	3	0.0%

TABLE 4

Data relative to fertility of artificial inseminations and control matings, series IV.

SERIES NUMBER	ESTIMATED SPERM PER INSEMINATION	TOTAL NO. OF INSEMINATIONS	NO. OF FEMALES INSEMINATED	NO. OF PLUGS	LEAKAGE, INFERTILE	PLUG IN P.M.			PLUG IN A.M.		
						Fertile	Infertile	Percent fertile	Fertile	Infertile	Percent fertile
IV a	4,000,000	43	31	24	3	10	4	71.5%	4	3	57.2%
IV a	800,000	49	37	30	6	13	5	72.3%	2	4	33.3%
IV b	3,500,000	35	26	18	2	8	3	72.7%	2	3	40.0%
IV c	1,750,000	16	16	16	2	9	2	81.8%	2	1	66.7%
IV d	2,000,000	10	9	8	0	0	4	0.0%	1	3	25.0%
Total	....	153	119	96	13	40	18	69.0%	11	14	44.0%
Control	....	..	..	47	..	31	3	91.2%	6	7	46.2%

Within the range used, the number of sperm per insemination did not affect the fertility. Series IVa was designed to test this point, the females each week being divided into three equal groups, one to receive the smaller number of sperm per insemination, the second to receive the larger, the third as controls. The animals receiving the lower number of sperm showed a fractionally higher fertility percent (72.3% as compared with 71.5%).

The quality of the sperm may have been a factor influencing the fertility percent in a few cases. We have always found that a high proportion, usually more than 50%, of sperm from the vas deferens is immotile. On two consecutive days in series IV the motility at the close of the inseminations was unusually low. The fertility percent on these 2 days was 50% for P.M. plugs (12 matings) and 0% for A.M. plugs (3 matings). During a period in series IV extending from March 27th to May 4th all sperm suspensions showed a very unusual degree of clumping. This was not altered by the preparation of fresh medium. There is no clear evidence that the fertility percent was effected either in the test or the control animals during this period.

During series III and for 2 weeks in series IV the tuberculin syringe containing the sperm suspension was kept in Locke's at 36.5°–37.5°C. between inseminations. Throughout the remainder of series IV, the sperm suspension was kept at room temperature. No affect of the temperature differences was noted.

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SUMMARY

1. A colony of Swiss X B alb C hybrid females was kept in a dark-room with lights on at 10:30 P.M., off at 10:30 A.M. Following weaning of litters, smears were taken daily until estrus was indicated. Females presumably in estrus were inseminated at about 4 P.M. with the aid of an especially designed speculum, a head mirror, and a blunt pointed no. 19 needle and tuberculin syringe. Turning the point to the right or left resulted in entry into the right and left horns of the uterus respectively. Following insemination, females were placed with vasectomized males and checks made for plugs at 5–6 P.M. and again the next morning.

2. The fertility percent following P.M. plugs was 69.0% (58 plugs); following A.M. plugs, 44.0% (25 plugs). Thirteen cases in which leakage of the injected sperm suspension occurred immediately following insemination are excluded. The corresponding figures for the control are 91.2% (34 plugs) and 46.2% (13 plugs).

3. There is a clear mode in the onset of estrus at 4 to 6 hours after the onset of darkness.

4. B alb C females under normal lighting conditions showed a fertility of 84% (38 plugs) at the second post partum estrus (the last litter

being killed at birth) and 95% (38 plugs) at the first post weaning estrus.

5. The condition of the cervix as observed during inseminations is in some cases a more reliable indication of the estrous condition than is the vaginal smear. Absence of moisture, tension of the cervix muscles, and absence of leakage following insemination are accompaniments of readiness to mate.

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OBSERVATIONS ON THE RATE OF TRANSPORT OF SPERMATOZOA IN THE FEMALE GENITAL TRACT OF THE RAT[1]

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In view of the extensive utilization of the rat in studies of reproductive physiology it is important to have complete knowledge of all events occurring in the normal course of insemination and fertilization. Hartman and Ball ('30), Warren ('38), and Rossman ('37) have conclusively demonstrated that within a few minutes after ejaculation the spermatozoa of the rat are disseminated throughout the uterine cornua. This rapid dispersion is brought about primarily by mechanical means (Rossman, '37), and can be explained by the presence of large quantities of uterine fluid and marked muscular activity of the cornua at the time the female will accept the male. The distance spermatozoa must travel through the cornua is approximately one-half that through which they must pass to arrive at the site of fertilization in the oviducts. The short interval necessary for sperm to reach the utero-tubal junction is no indication of the transport rate through the oviducts. In the rat there are no data on the rapidity of sperm transport from the moment of ejaculation to the time of arrival at the tubal or periovarian sacs. It was considered important, therefore, to undertake such a study.

MATERIALS AND METHODS

The data in this study were obtained from fifty-eight female rats between 3 and 4 months of age, which were bred from the Brown University strain. The onset of sexual receptivity was ascertained by the animal's response to manual manipulation as described by Blandau, Boling and Young ('41). Individual females, in heat, were placed with vigorous males and observed continuously to determine the precise moment of ejaculation.

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When ejaculation had taken place the male was removed from the cage and the female was allowed to remain undisturbed until the time for examination in order to reduce the possibility of failure of insemination (Hartman and Ball, '30). The inseminated females were killed by decapitation at intervals of 15, 30, 45, and 60 minutes, respectively, following the observed insemination. Immediately after the female was killed, she was opened ventrally and haemostats were applied: (1) immediately above the cervix, (2) in the approximate center of each cornu, (3) just above the utero-tubal junction, and, (4) on the fourth or fifth loop of each Fallopian tube. A clean set of instruments was used for each dissection so as to eliminate the possibility of sperm transfer from one segment to the next. The uterine segments were removed separately, flushed with Locke's solution, and the latter examined for spermatozoa. The coiled oviducts may be divided into a proximal and distal portion of four or five folds each. The distal portion communicates with the periovarian space while the proximal segment opens into the cornu. For the sake of clarity the usual anatomical divisions of the oviduct into proximal and distal segments will be abandoned in favor of the terms ovarian and uterine segments. Each segment was separately straightened by trimming away the mesosalpinx with a pair of iridectomy scissors. The contents of the tubes, expressed into separate receptacles by stroking their lengths with a probe, were subsequently examined for spermatozoa.

OBSERVATIONS

Large numbers of freely swimming spermatozoa were observed throughout the uterine cornua of all animals examined 15 minutes or longer after ejaculation.

Table 1 summarizes the essential data and indicates that within 15 minutes after ejaculation spermatozoa had already migrated into the uterine segments of ten of the twenty-four oviducts examined. In five

TABLE 1

The relative rates at which spermatozoa reach the uterine and ovarian segments of the oviduct.

TIME, IN MINUTES, BETWEEN EJACULATION AND KILLING OF FEMALE	NUMBER OF OVIDUCTS	NUMBER OF OVIDUCTS IN WHICH SPERM HAD REACHED THE UTERINE SEGMENT			NUMBER OF OVIDUCTS IN WHICH SPERM HAD REACHED THE OVARIAN SEGMENT		
		Right	*Left*	*Percent*	*Right*	*Left*	*Percent*
15 min.	24	5	5	42	3	2	21
30 min.	42	19	18	88	13	13	62
45 min.	30	14	13	90	13	13	86
60 min.	20	10	10	100	10	10	100

of the twenty-four oviducts spermatozoa were present in the ovarian segment. Because it was impossible to place the haemostats on the exact center of the coiled oviducts, too much importance should not be applied to the division of the segments. Rather, the emphasis should be placed on the observation that spermatozoa were passing through the oviducts in 42% of those examined at this time.

At 30 minutes after ejaculation, spermatozoa were found in the uterine segments in 88% of the forty-two oviducts examined. In twenty-six of these, spermatozoa had migrated past the arbitrary midpoint of the oviduct into the ovarian segments.

Spermatozoa had failed to enter only three of the thirty oviducts examined 45 minutes after ejaculation, and in 86% of the thirty oviducts spermatozoa had migrated into the ovarian segments.

At 1 hour after ejaculation 100% of the twenty oviducts examined showed spermatozoa throughout the length of the tubes.

In a number of animals the concentration of spermatozoa was notably less in one than in the other uterine cornu. This observation is of interest in that it is contrary to the belief that approximately equal numbers of spermatozoa enter the cornua at the moment of ejaculation. In an animal examined 30 minutes after ejaculation it was noted that the sperm concentration was distinctly less in the left cornu when compared to the right. The ovarian segment of the right oviduct contained approximately twenty spermatozoa. Three spermatozoa had migrated into the uterine segment of the left tube while none were found in the ovarian segment of the same oviduct. A difference in sperm concentration in the cornua may account for variations not only in the rate of transport but also in the number of sperm reaching the ampullae of either of the oviducts of the same animal.

On the other hand estimations were made of the number of spermatozoa migrating through the oviducts at the various time intervals studied. At 15 minutes after ejaculation the oviducts rarely contained more than fifteen to thirty spermatozoa. This number gradually increased during the later intervals until a maximum number of 200 or more spermatozoa were obtained from a single tube in several animals killed 45 to 60 minutes after ejaculation. However, there was great variation in the number of spermatozoa present from animal to animal and in oviducts of the same animal at each of the intervals studied.

DISCUSSION

In the rat the temporal relationships between sexual receptivity, ovulation, changes in the cervix and uterus, and the arrival of spermatozoa at the site of semination are so synchronized that reproduction is remarkably efficient.

Hartman and Ball ('30) and Genell ('39) have demonstrated that spermatozoa are catapulted into the uterine cornua at the moment of ejaculation. During heat the uterine cornua are markedly distended with a secretion which Kugota ('29) and Warren ('38) have shown to be favorable to the motility of spermatozoa. The marked peristaltic and antiperistaltic muscular activity of the cornua during heat (Rossman, '37) churns the contents in such a manner that spermatozoa are disseminated within a few seconds. Their independent movement is thus a negligible factor insofar as their ascent through the uterus is concerned. The factors involved in further transport through the oviducts have not been elucidated. In this study we have shown that in a number of animals within 15 minutes after the spermatozoa have entered the cornua, they can be found to have migrated throughout the oviducts and to have reached the ampullae. Spermatozoa will be found in the ampullae in 100% of the animals examined 1 hour after ejaculation. The reproductive processes in the rat are so timed that spermatozoa are present in the Fallopian tubes and are ready to fertilize the ova immediately upon their extrusion from the follicles. It is obvious that this sequence of events is important when we realize that rapid devitalization of the ova occurs if fertilization is delayed (Blandau and Jordan, '41).

There is considerable unanimity in the results described above with those obtained by Lewis and Wright ('35) for the mouse. In an animal killed 15 minutes after ejaculation a few active sperm were found in the first and second loops above the utero-tubal junction. In a second animal killed 40 minutes after insemination two active sperm were found in the first loop above the eggs and none in the periovarian sac. Thus in the mouse the rate of sperm transport compares favorably with that which we have observed in the rat, and the number of spermatozoa entering the oviducts in both species is remarkably few in view of the large numbers in the ejaculate. The mouse uterus, during estrus, is also greatly distended with fluid and thus the rapid transport of sperm may be the result of both direct insemination and uterine motility as described for the rat.

In the guinea pig sperm have been found in the uterus in 5 minutes and in the middle of the oviducts in 15 minutes after coition (Leuckart, 1853).

Sperm transport in the dog appears to be even more rapid. They are probably carried en masse by the antiperistaltic waves passing up the uterus (Bishoff, 1845, and Evans, '33), and may appear in the upper ends of the Fallopian tubes as early as 18 minutes after ejaculation (Whitney, '27).

The rate of sperm transport is not this rapid in all species. In the rabbit the time necessary for spermatozoa to reach the infundibulum is considerably longer than that reported for the rat, the mouse, or the dog, requiring 1½ to 2 hours to reach the utero-tubal junction and approximately 2 hours more to reach the infundibulum (Parker, '30; Heape, '05; Pincus, '30). Green and Winters ('35) and Quinlan and Maré ('31), found that 5 to 6 hours are necessary for sperm to reach the infundibulum in the ewe, the rate depending on the length of the female tract and the activity of the spermatozoa.

Hartman ('39), has summarized the factors which may be important in accounting for the wide variation in the rate of sperm transport in different animals. Most consideration has been given: (1) the action of cilia, (2) the muscular activity of the uterus, vagina and oviducts, and (3) the locomotor activity of the spermatozoa themselves. There are many other factors, however, which need investigation. There is much speculation but little definite information concerning the part played by the cervix or utero-tubal junction in sperm transport. In addition, recent observations seem to indicate that the female orgasm and the vaginal plug are of importance in insemination of the rat and probably of other rodents.

SUMMARY

Fifty-eight female rats were killed after insemination at intervals of 15, 30, 45 and 60 minutes respectively and their reproductive tracts examined for spermatozoa.

Spermatozoa had migrated into the uterine segments in 42% of the twenty-four oviducts examined 15 minutes after ejaculation. In 21% of the twenty-four oviducts spermatozoa had reached the ovarian segments.

At 30 minutes after ejaculation, spermatozoa were found in the uterine segment in 88% of forty-two oviducts examined. In 62% of the forty-two oviducts spermatozoa had reached the ovarian segments.

In only three of thirty oviducts examined 45 minutes after ejaculation had spermatozoa failed to pass the utero-tubal junction. Spermatozoa had migrated into the ovarian segments in 86% of the oviducts examined at this interval.

Twenty oviducts examined 1 hour after ejaculation showed spermatozoa throughout.

The number of spermatozoa entering the oviducts is exceedingly small when compared to the number present in the cornua.

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SELECTIVE STAINING OF GLYCOGEN BY AMMONIACAL SILVER NITRATE: A NEW METHOD

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FIVE FIGURES

During an investigation on the use of the Pap's silver method for the demonstration of reticulum following a variety of fixatives, it was discovered that, with a minor modification, this method offered a valuable means for the selective staining of glycogen. It is the purpose of this communication to present this new procedure for the demonstration of histological glycogen.

The tissues utilized consisted of the livers of guinea-pigs and placentae of rats, guinea-pigs, cats and pigs. The sections stained by silver were compared with equivalent sections stained by Best's carmine and the Bauer-Feulgen method. The efficiency of the method was also tested by exposing the glycogen in control sections to digestion with saliva.

FIXATION

Picric acid, alcohol, formaldehyde fixative:

Picric acid, sat. sol. in absolute alcohol	90 cc.
Neutral formaldehyde	10 cc.

Fix from 6 to 12 hours. Wash in absolute alcohol with several changes; wash in chloroform and absolute alcohol with several changes and transfer to chloroform. Embed in paraffin.

STAINING

The method here employed is derived by slight modification from one introduced by Pap ('29) for the impregnation of reticulum fibers.

The deparaffinized sections are treated as follows:

1. Place in 0.25% aqueous potassium permanganate — 5 to 10 minutes.
2. Rinse in distilled water — 1 to 2 minutes.
3. Decolorize in 5% oxalic acid — 5 minutes.
4. Rinse in distilled water.
5. Place in 2% aqueous silver nitrate — 12 to 24 hours.

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6. Transfer to 1% ammoniacal silver nitrate — 15 to 30 minutes.
7. Rinse in 4% neutral formaldehyde — 5 to 20 seconds.
8. Rinse in running water — 1 minute.
9. Tone in 0.2% gold chloride — 5 to 10 minutes.
10. Rinse in running water.
11. Fix in 5% aqueous sodium thiosulphate — 5 to 10 minutes.
12. Wash in running water — 1 minute.
13. Counterstain, if desired, with Mayer's paracarmine.
14. Dehydrate, clear in xylol, and mount in balsam.

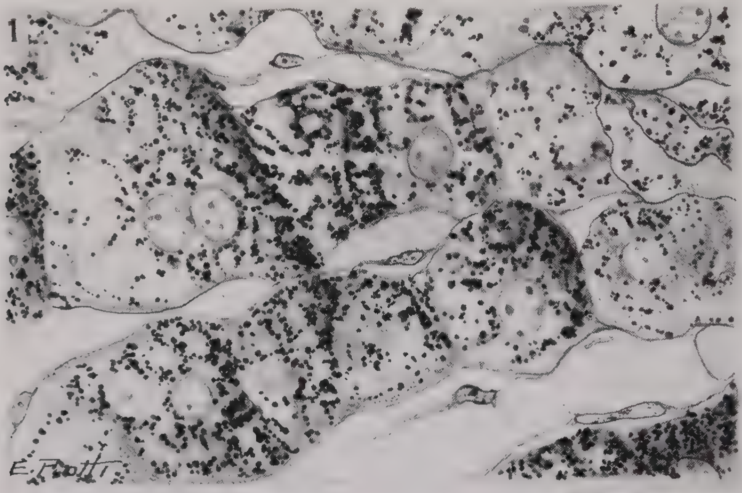

Fig. 1 Parenchyma of the liver of a guinea-pig showing the hepatic cells laden with glycogen which has been impregnated by reduced ammoniacal silver nitrate. Counterstained with Mayer's paracarmine. × 1600.

The ammoniacal silver nitrate solution (step 6) is prepared as follows: 1 gm. of silver nitrate is dissolved in 10 cc. of distilled water. To this are added 11 drops of 40% aqueous solution of potassium hydroxide. The resultant precipitate is dissolved by adding 26% ammonia, drop by drop, and absolute alcohol is added up to a total of 100 cc. The solution is allowed to stand over night and used for step 6.

The only departure from Pap's original method is in the preparation of the ammoniacal silver nitrate solution. Instead of diluting with distilled water in the final step, we dilute with absolute alcohol, a procedure which prevents the sections from coming off the slides in the subsequent steps of the staining method.

RESULTS AND COMMENT

We have obtained excellent results by this procedure when the recommended methods of fixation and staining have both been rigorously adhered to. The intracellular material which we assume to be "glycogen" stains an intense black (figs. 1 and 5). Our evidence for assuming that the stained material within the cytoplasm of the cells is glycogen rests upon comparison with similar sections stained by Best's carmine and the Bauer-Feulgen method. The distribution of the glycogen revealed by our method coincides with that following the two older procedures (figs. 3, 4 and 5). Moreover, analogous to the two previous methods, no intracellular silver deposits occur when control sections have first been treated with saliva.

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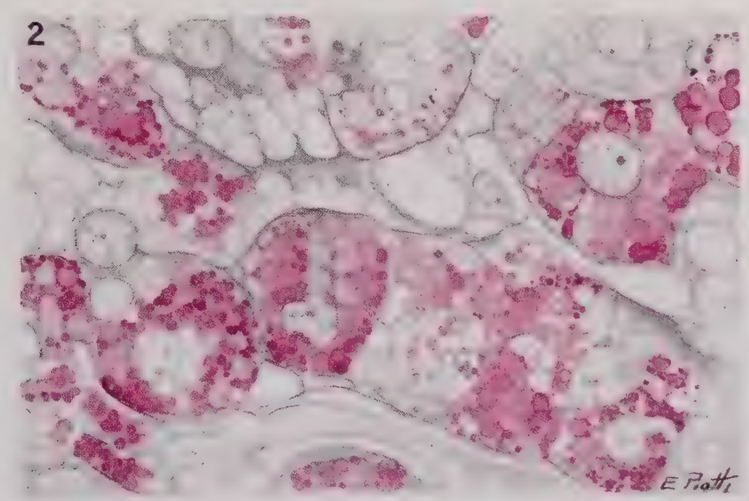


Fig. 2 A similar section of a guinea-pig's liver stained by the Bauer-Feulgen method. × 1600.

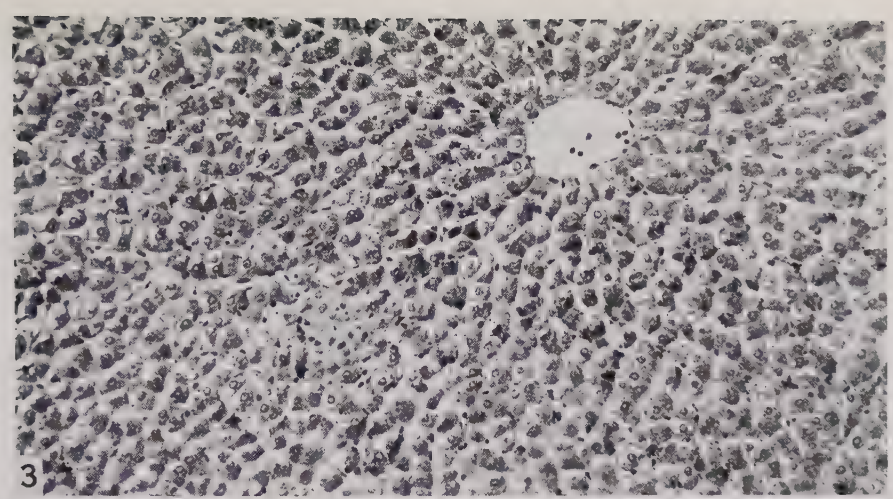
We have not made a survey by our method of all mammalian tissues known to contain histological glycogen but have limited ourselves to examination of the liver of the guinea-pig and of the placenta of a variety of animals. In both of these tissues, the glycogen demonstrated by our method coincides exactly with deposits stainable by Best's carmine and the Bauer-Feulgen method.

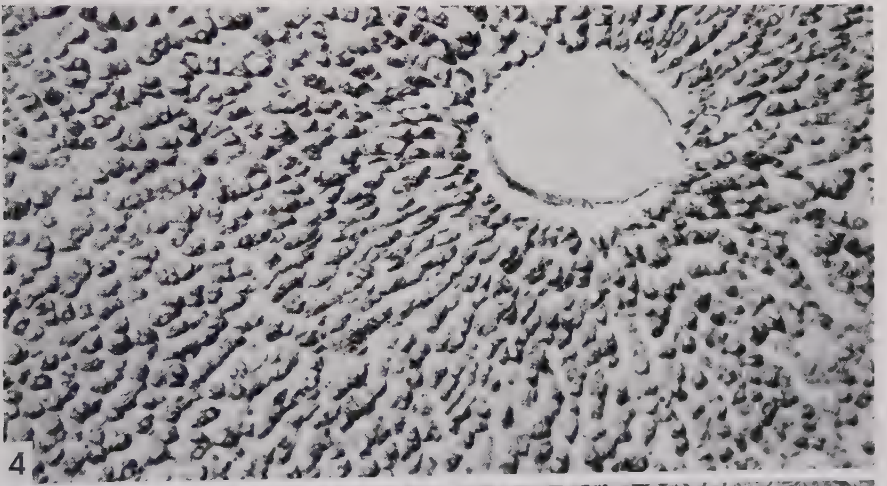
For the above reasons we are confident that our procedures represent a selective method for the demonstration of histological glycogen. We are not familiar with any similar previous use of silver for this purpose. The advantages and disadvantages of the present method are briefly presented. We find the glycogen to be more intensely and more consistently stained than by either of the other methods. Even the smallest aggregates and particles of glycogen, seen at high magnification, are sharply delineated by the silver method, whereas by the procedures of Best and Bauer-Feulgen the smallest aggregates seem to be indistinctly and hazily stained, and the borders of the larger aggregates have a fuzzy or washed-out appearance. By the silver technique the borders are much sharper and the masses, both large and small, are much more discrete. These differences in the finer details between our method and the previous techniques are illustrated in figures 1 and 2 showing glycogen in cells of the guinea-pig's liver under the oil immersion lens. The density and sharpness of the blackened material, especially the smallest particles, suggest that the glycogen is probably more completely stained than by other existing methods.

A certain practical advantage of the present method is that the glycogen appears black in the sections, and hence lends itself readily to black and white reproduction either as drawings or by photography.

The only drawback to our method for certain purposes that we can foresee, is the fact that in addition to the glycogen the method also stains selectively the reticulum fibers of the connective tissue. This is an obvious disadvantage when it is desired to study the finer distribution of glycogen in the reticular connective tissues. In these cases a certain amount of confusion arises as between the exact amount and location of glycogen in the connective tissue cells which are intimately lodged in a delicate network of blackened reticulum fibers. We have met with this difficulty in attempting to arrive at fine discriminations regarding glycogen in fibrillar decidual tissue and in fetal mesenchyma associated with placental tissues. On the other hand, in the great variety of epithelial tissues which are not intimately associated with reticular fibers, the silver method offers a very favorable and consistent technique for the study of glycogen. Besides in the hepatic parenchyma, we have found it an excellent method for the demonstration of glycogen in the vitelline epithelium of the rodent's placenta, in the giant cells of the rat's placenta, and in the chorionic epithelium of the pig's placenta. Although we have not tried it in other localities, it should prove useful in staining glycogen in cartilage cells, as well as in embryos where glycogen is widespread in many of the epithelial tissues.

We do not wish to discuss the moot question of the nature of the histological staining methods for glycogen. Suffice it to say regarding the present method that its success depends upon the use of picric acid in the fixative. Plain alcohol, or alcohol and formaldehyde followed by





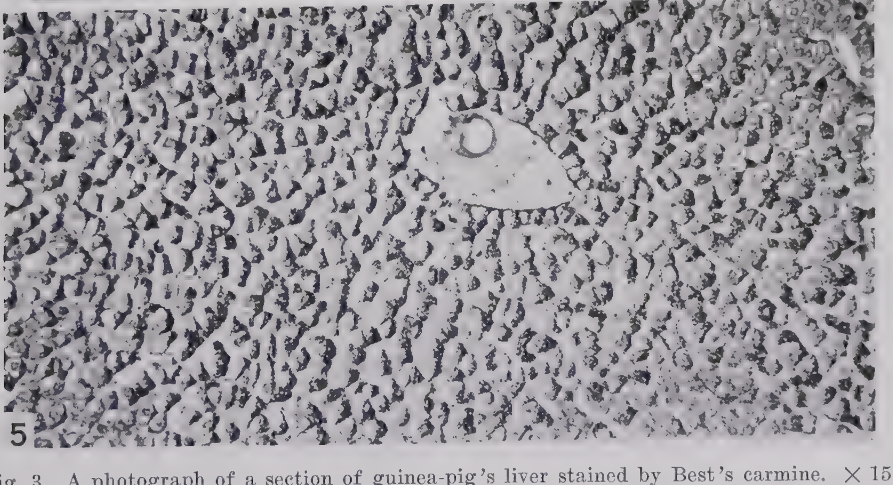


Fig. 3 A photograph of a section of guinea-pig's liver stained by Best's carmine. × 150.

Fig. 4 A similar section stained by the Bauer-Feulgen method for glycogen. × 150.

Fig. 5 A similar section showing the hepatic glycogen impregnated by silver nitrate according to the present method. × 150.

the silver method have given unsatisfactory results. Thus the success of the method depends in good measure upon the picric acid which is a recognized fixative for proteins. Apparently a combination of such a protein fixative (picric acid) and a preservative of glycogen (alcohol) is requisite for favorable reduction of the ammoniacal silver by the aldehyde groups resulting from hydrolysis of glycogen.

Corrosive sublimate in combination with alcohol and formaldehyde as a fixative for glycogen followed by Best's stain was recommended to one of us many years ago by Dr. E. W. Goodpasture and had been tried at the time with success. We have repeated this recommendation, utilizing 5% corrosive sublimate in the fixative, with good success as far as Best's stain is concerned, but with very indifferent results following the application of the ammoniacal silver nitrate. Thus for the subsequent successful reduction of the silver solution the substitution of mercuric chloride for picric acid has proved ineffective, suggesting that the action of the sublimate upon the mixture of proteins and glycogen in the cells differs from that exercised by picric acid.

We have achieved such satisfactory results by adapting Pap's method to our purpose that we have not explored the possible use of any of the other closely related methods of impregnation with ammoniacal silver nitrate. It is more than likely that related silver impregnation techniques will produce similar results.

We wish to express our appreciation to Dr. H. L. Weatherford for his helpful advice regarding the method and material.

SUMMARY

A new method is described for the selective staining of glycogen. It involves fixation in picric acid, alcohol and formaldehyde, and subsequent staining of the sections by a modification of Pap's ammoniacal silver method for the impregnation of reticulum.

The results obtained by this method are compared with Best's carmine stain and the Bauer-Feulgen procedure for the demonstration of glycogen.

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AN EXPERIMENTAL STUDY OF FACTORS INFLUENCING THE COURSE OF NERVE FIBERS IN THE EMBRYONIC CENTRAL NERVOUS SYSTEM[1]

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THREE TEXT FIGURES AND THREE PLATES (SIXTEEN FIGURES)

The problem of ascertaining the factors which control the direction taken by growing nerve fibers, and consequently the forces influencing development of the normal pattern of pathways in the central nervous system, has long been a matter of speculation and controversy. Theories propounded to answer this question fall into three main categories. The mechanical theory, first proposed by His in 1887, lays emphasis upon arrangement of the ground substance, asserting that nerve fibers follow available paths in this substrate. The chemotropic theory, advanced by Cajal in 1893, attributes specific chemical characteristics to individual regions for which particular groups of nerve fibers have an affinity. The theory of galvanotropism, formulated by Strasser in 1892 and elaborated by Kappers ('17), as the theory of neurobiotaxis, holds that differences of electrical potential between regions cause axons to grow with the current and dendrons to grow against it.

In support of the theory of neurobiotaxis Bok ('15) proposed a theory of stimulogenous fibrillation in embryos. He claimed that the descending medial longitudinal fascicle initiated development of the cranial motor nuclei, causing the first axonal processes of neighboring neuroblasts to grow away at right angles from this tract. According to his observations the first cephalocaudal wave of development affected sequentially the trochlear, trigeminal, facial, glossopharyngeal and vagus motor nuclei. He reported a similar wave of development appearing a day later to initiate development of the oculomotor, abducens and hypoglossal nuclei.

The chemotropic and galvanotropic theories were supported by few experimental investigations. All of the earlier studies were in the realm of normal comparative anatomy and embryology. Most of the experi-

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[1] Aided by a grant from the John and Mary R. Markle Foundation.

mental work which has subsequently been done has supported the mechanical theory (Harrison, '35; Weiss, '39). The most significant experiments have been concerned with the peripheral nervous system or with growth of neuroblasts in tissue culture rather than with the central nervous system. None has dealt specifically with the development of tracts within the central nervous system. The experiments which are presented in the present report deal with changes in central nerve pathways following surgical alteration of the neural tube of the early embryo.

MATERIAL AND METHODS

Hen's eggs were incubated at 99°F. for periods of 38 to 40 hours at which time 17 to 20 somites had formed. Neurofibrillar development is just starting at this time (Windle and Austin, '36).[2] The position of the germ disc was determined by candling the eggs at that time. About 2 cc. of albumin were withdrawn from a hole in the small end of the egg with a hypodermic syringe. Following this a hexagonal window about 1 cm. in diameter was cut in the shell directly over the germ disc. The operations were performed under a wide-field binocular microscope. Illumination was provided by a beam of light transmitted through heat-absorbing glass.

A vibrating knife operated by 60-cycle alternating current similar to that devised by Drury ('41), was found useful to excise small sections of the brain and to produce gaps in the neural tube. The cutting blade of the knife was constructed from an embroidery needle, size 11. For removing the rostral end of the neural tube a constrictable hair loop was placed around the free end of the embryo's head. Closure of the loop cut off the part of the head in front of it and this could then be removed by lifting it out with a spatula. The window was closed after the operation by sealing a piece of glass over the opening of the shell with a rim of wax. The eggs were then replaced in the incubator and development was allowed to continue until the desired age had been reached. Embryos were then removed and fixed in ammoniated alcohol for the pyridine-silver method outlined by Davenport, Windle and Beech ('34).

Operations were performed on a large number of chicks at the levels indicated in figures 1, 2 and 3. For this study forty-seven embryos of 4 to 9 days' incubation were selected because of excellence of the silver stain and favorable planes of sections. The forebrain had been removed in fifteen of them. Twenty-two had been deprived of all parts of the brain rostral to the trigeminal region. Complete gaps had been produced in the midbrain or hindbrain of nine embryos. In one the tectum had been removed and the basal part of the midbrain was intact on one side only. Thirteen normal chick embryos of comparable ages and sectioned in similar planes were used for control.

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[2] At 18 somites neuroblasts have begun to differentiate at the level of the otic invagination and at the junction of mesencephalon and diencephalon. No tracts or commissures have been formed.

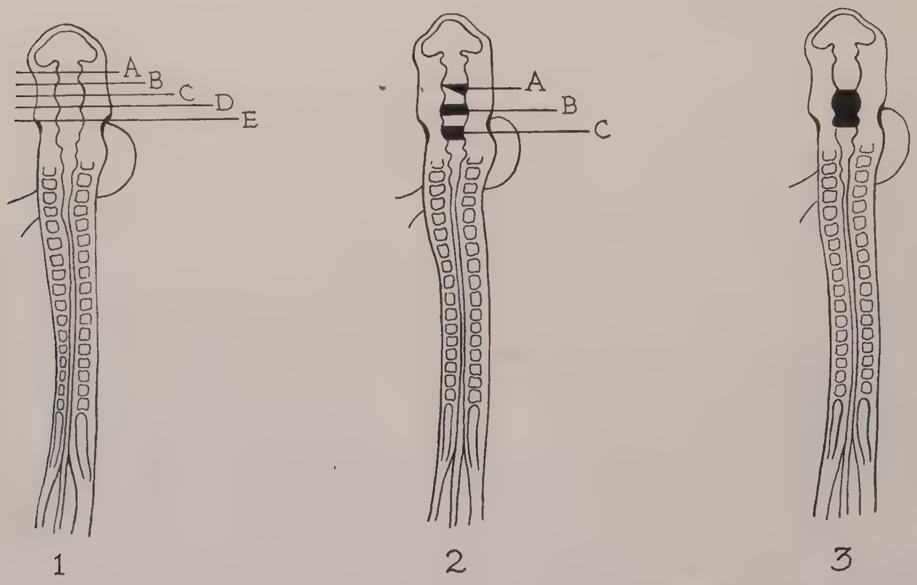
OBSERVATIONS

The histologic picture of the brain varied considerably with the level at which the operation was performed. Regardless of where the cut was made or what part of the brain was removed at operation those portions of the nervous system below the cut went on to develop a structure which was almost indistinguishable from that characterizing the normal intact embryo at the same level. This was demonstrated in the illustrations of a previous article (Rhines, '43). The same applies to parts in front of a gap produced by removal of a portion of neural tube. Removal of portions of the brain did not even retard development significantly unless the level of the cut was made as low as the otocyst. In the latter case retardation of 24 to 30 hours at most was observed.

Removal of the rostral portion of the brain at 38 to 40 hours of incubation was always followed by closure of the wound and sealing off of the brain ventricle. If the level of the cut was through or above the mesencephalon the tectum formed a blister-like cap over the rostral end of the brain. In the older embryos of the series the head appeared quite abnormal due to the absence of the eyes and the rostral part of the brain. The upper bill was always missing.

Alteration of normal fiber pattern at operation site

In specimens from which the rostral portion of the brain had been removed (fig. 1) the closed stump of the brain lay close to the ectoderm and was separated from it by only a small amount of mesenchyme. In a group of embryos in which the level of section was through the metencephalon (fig. 1, D and E) a characteristic rearrangement of the fiber pattern was constantly observed. Usually the end of the brain was flexed ventrally (fig. 4). In some cases the sensory ganglia were located ventral and rostral to the neural tube and the sensory roots entered the rostral end of the brain. Internal alterations were present at the end of neural tube but a very short distance caudally there was a transition to structures of normal appearance. In the altered region no longitudinal groove occurred in the very thick floor plate. The latter structure formed a hump which caused the ventricle to appear as a flattened, dorsally convex crescent, like that seen in figure 6. At either horn of the crescent-shaped ventricle the junction of alar and basal plates occurred. Completely closing off the rostral end of the ventricle was a rounded terminal cap of neural tissue composed of ependymal, mantle and marginal layers. In the ventral part of the neural tube the marginal layer continued caudally on either side as the reticular formation of the basal plates. Medially it was replaced by the floor plate which extended almost to the rostral end of the neural tube.



Figs. 1–3 Diagrams of the 38-hour (17-segment) chick embryo. Figure 1 shows the levels rostral to which the free part of the head was removed surgically. Figures 2 and 3 illustrate the locations and extent of excised segments of the chick brains.

Sensory tracts ascending near the sulcus limitans followed the curve of the dome-shaped brain stump and decussated to form an arch in the marginal layer as shown at the top of figure 4. Scattered bundles of ascending fibers of the reticular formation built arches in the mantle layer. More internally a similar decussation was formed by the medial and ventral longitudinal fascicles as they curved around the rostral end of the floor plate. This is best seen in figure 5. It could not be determined with certainty whether the arching ascending fibers actually descended for any distance on the opposite side after reversing their direction. Most of the embryos in which this decussation was clearly seen, i.e., those with the brain ending in the metencephalon, were incubated only 4½ to 5½ days, 3 to 4 days beyond the time of operation. In two embryos of 6 days' incubation the arching ascending fibers met in or near the midline and formed bundles which emerged from the rostral end of the brain rather than descending on the opposite side (fig. 11). A description of the emergence of central fibers will be found on pages 273 to 277.

In the curving marginal layer of the brain stump pyriform neuroblasts were oriented radially with their long axes perpendicular to the curve of the end of the brain. Their axons converged toward the midline like spokes of a wheel and followed a straight, diagonally caudal course through the reticular tracts and the medial and ventral longitudinal fascicles to enter the floor plate. There the fibers met and crossed, forming the hub of the wheel, and continued caudad and laterad, as the spokes, to descend in the medial longitudinal fascicle or the reticular formation. These structures are shown in figures 4, 5 and 6. Some of the fibers originating in the sides of the wheel-like arrangement turned rostrad in the medial longitudinal fascicle after crossing. There was a tendency for axons of neuroblasts located in the midline at the top of the wheel to continue caudad in the floor plate. In two embryos these fibers actually formed prominent longitudinal tracts in the floor plate like that at the arrow in figure 9. These continued for some distance below the region of abnormality and ended in the floor plate.

Brains terminating in the mesencephalon or at the junction of mesencephalon with diencephalon (fig. 1, A, B and C) presented the same picture of ascending tracts arching across the blind end of the neural tube. Occasionally a wheel-shaped commissure was formed by neuroblasts in the vicinity. This fiber arrangement was more pronounced in older embryos (8 to 9 days) because arrival of the ascending tracts and development of the commissures occurred later in the rostral part of the mesencephalon than in lower parts of the brain.

In two embryos the myelencephalon was separated from the metencephalon by a gap (fig. 2, C). Alterations produced at the end of the myelencephalon were strikingly different from those seen in embryos with more rostral lesions. The altered region included all of the glossopharyngeal and vagus level. Transverse sections of the brain were open dorsally like the primitive neural tube before approximation of the neural folds. Furthermore, differentiation of longitudinal plates and of ependymal, mantle and marginal layers was not apparent. Consequently the neural tube had a homogeneous and primitive appearance. The ventral part of the neural tube was thin and undifferentiated although fibers formed a U-shaped decussation in its peripheral part. In spite of the primitive appearance of the myelencephalon there were many fibers in it. Near the gap bundles of sensory fibers from vagus and glossopharyngeal ganglia entered ventrally. No specific pathways except the solitary tract were identifiable. The brain assumed its normal pattern in the caudal part of the myelencephalon coincident with appearance of the floor plate. At the rostral end of the floor plate a radial commissure occurred. In one embryo this region closely resembled the end of the brain in specimens sectioned through the metencephalon. Ascending from the normal region, small ventral longitudinal fascicles arched over the end of the floor plate; the latter structure terminated a considerable distance from the end of the brain.

Occasionally local alterations in the course of central fibers were observed. In two embryos fibers crossing in an apparently normal commissure turned longitudinally in the middle of the floor plate (fig. 7). This occurred adjacent to a pocket which developed in the floor plate a short distance from the site of an incision through the brain. In two other embryos with gaps produced in the brain caudal to the trochlear nerve level (fig. 2, B and fig. 3) the normally homolateral diencephalic components of the medial longitudinal fascicles entered into the formation of a partial commissure in the rostral stump just caudal to the trochlear nuclei where in both instances the floor plate ended. A commissure of this type is illustrated in figure 8. In neither embryo was the decussation located at the immediate site of the operation but was a little rostral to it. Some of the nerve fibers resumed their caudal course after crossing. In one of the specimens they formed two small bundles on either side of the midline below the ventricle. The floor plate appeared to be absent from this point caudally to the end of the rostral stump. The bundles of nerve fibers ended by taking a transverse course at the end of the stump. But instead of curving toward each other they both turned toward one side, merged into one fascicle and ended within the brain. The brain was quite symmetrical in the region where the fibers were coursing longitudinally but at the end of the stump became asymmetrical.

In the other embryo the brain extended for a considerable distance caudal to the trochlear level. It was rather shapeless and the fiber pattern was badly disorganized. Many nerve fibers of the diencephalic components of the medial longitudinal fasicles descended in scattered bundles after meeting at the midline or after crossing there to form commissures. The descending bundles did not have the characteristic appearance of medial longitudinal fascicles and could be identified only by tracing them back to the region of the commissure. In this specimen as well as in the former one, other descending nerve fibers formed decussations across the rostral stump of the brain.

Emergence of central fibers at operation site

Abnormal emergence of fibers was observed in twenty-eight of the forty-seven operated embryos of the present series. Groups of central nerve fibers passed out of the brain usually only at the site of operation or very near it. The emergence of fibers and the paths they took were more variable than the formation of decussations in the brain at the site of operation.

The forebrain, midbrain and rostral part of the hindbrain were removed in twenty-two embryos (fig. 1, D and E). Central tract fibers emerged from the brain and passed into the mesenchyme in eleven of these specimens. In six of them bundles of axons from neuroblasts of the reticular formation escaped from the ventral part of the brain stump. In most instances the fibers took an irregular, apparently aimless course in the mesenchyme occasionally reaching the neighboring ectoderm. In one embryo a small bundle of fibers joined the maxillary branch of the trigeminal nerve. In another a fiber bundle passed caudad to join the abducens nerve. After uniting with cranial nerve bundles central tract fibers lost their identity and their extent could not be determined.

Ascending fibers of primary afferent tracts, reticular formation and medial longitudinal fascicles passed out of the rostral tip of the brain stump and formed a thick bundle in one embryo illustrated in figure 11. This reached the nearby ectoderm giving off a small branch to the mesenchyme before entering the ectoderm. The structural alterations of the brain stump of this embryo were similar to those described in the preceeding section of this article except that the arching ascending fibers failed to cross the midline. Instead they met and turned rostrad. At the end of the brain the pia mater was lifted up forming a small cyst along the side of which the emerging fibers traveled as they entered the mesenchyme.

In another embryo with the brain removed at the same level ascending central fibers turned rostrad and left the brain but remained in the pia mater ending therein after a short course. Both of these specimens were 6 days old and were the oldest in the present group. Fibers emerged from the floor plate in three other embryos at the region of transition from a wheel-shaped commissure to one of normal appearance. Many commissural fibers converged in the floor plate and formed a bundle which left the brain ventromedially and continued in a ventral course until it ended near the notochord (fig. 14). This type of aberrant central tract occurred in the caudal segments of five brains having gaps and in one embryo with the forebrain absent. In the last embryo the emerging bundle occurred at the abducens level where no disturbance of normal fiber pattern could be observed. Usually some brain tissue accompanied a fiber bundle for part of its aberrant course.

In fifteen embryos the brain terminated in the mesencephalon or the caudal part of the diencephalon (fig. 1, A, B, and C). Eight of these showed abnormally emerging central nerve fibers. In this series the course of fibers was somewhat more complicated than in the preceeding group. The identity of most emerging fibers was difficult to establish. Probably many of them were ascending fibers of tectal origin although in one instance they appeared to arise from neuroblasts of the interstitial nucleus (medial longitudinal fascicle). In the latter specimen, an embryo of 4½ days' incubation, bundles of fibers from the interstitial nuclei emerged laterally on both sides at a point rostral to the oculomotor nucleus. Thus the diencephalic components of the medial longitudinal fascicles became peripheral nerves. They followed irregular courses in the mesenchyme and ended beneath the ectoderm. A few of the fibers from the interstitial nucleus remained within the brain and could be followed in the position characteristic of a normal medial longitudinal fascicle as far as the trochlear level. There ascending fibers of the medial longitudinal fascicle mingled with them.

In each of three older embryos (5½ to 9 days) a bundle of emerging central tract fibers formed a structure resembling a peripheral nerve. This fascicle ran parallel to the oculomotor nerve. In one, a 6-day embryo in which interstitial nuclei were retained in the rostral end of the stump, many central fibers emerged from the stump and passed into the mesenchyme from the region rostral to the oculomotor nuclei. None of these fibers came from the interstitial nuclei but most of them seemed to have their origin in the rostral part of the tectum which formed a cap over the termination of the brain stem. Fibers were quite disorganized in the rostral part of the brain stump and those which emerged made their exit laterally as well as ventrally. The more laterally emerging bundles, like those illustrated in figure 13, wandered into the mesenchyme where one reached the ectoderm and traveled for a short distance immediately beneath and in contact with it. In contrast to the nonspecific course of these lateral bundles those which emerged ventrally entered the mesenchyme close to the oculomotor nerve and the junction of the internal carotid and basilar arteries. The internal carotid was a median unpaired artery in most operated embryos (fig. 12). In one specimen the aberrant bundle of central fibers, having made its exit, applied itself to its ventral wall. Some of the nerve fibers of the trochlear nerve joined this median bundle. Ventral to and between the oculomotor nerves this bundle descended along the arterial wall almost to the region between the two semilunar ganglia, i.e., the region occupied by the extraocular muscles.[3] Just rostral to the muscles the bundle joined one oculomotor nerve.

A similar nerve-like structure was present in two other embryos of the series. In one the fascicle did not join the oculomotor nerve but passed directly to the nerve plexus among the extraocular muscles. In other respects these specimens were similar to the others described and only minor variations were observed.

The brain of an embryo incubated for 5½ days ended just rostral to the trochlear nuclei (fig. 1, C). Ventral to the upper part of the metencephalon, rostral to the trigeminal level a small, isolated fragment of brain tissue was left lying against the inner surface of the ectoderm. From the floor plate of the brain at this level a short, pointed tongue of brain tissue extended toward the isolated fragment. The medial longitudinal fascicles ascending from the intact portion of the brain turned sharply ventrad and entered the projection but ended without escaping into the mesenchyme. The extramedullary trochlear nerves approached one another at this point and one received a small bundle from the other. Afterward it coursed directly to the isolated brain fragment and entered it. The isolated piece of brain contained three small groups of neuroblasts. Two of these resembled the interstitial nuclei. Axons from them entered the mesenchyme. On one side the fibers ended beneath the neighboring ectoderm. On the other side the emerging fascicle split into two bundles one of which descended laterally in the mesenchyme finally merging with the mandibular branch of the trigeminal nerve. The other bundle descended in the mesenchyme near the midline to reach the extraocular muscles between the trigeminal ganglia. From the third cell group a fiber bundle entered the mesenchyme and likewise descended to the extraocular muscles. The course of the last two bundles was similar to that of oculomotor nerves in operated, eyeless embryos in which the identity of the oculomotor nuclei could be definitely established. Whether some of these neuroblasts actually were remnants of the oculomotor nuclei could not be determined. It seems probable, however, that at least some of them belonged to the interstitial nuclei. Another group of aberrant central fibers in this embryo emerged from the base of the brain at the trochlear level, entered the pia mater and descended for a short distance.

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[3] The superior oblique muscle was absent.

In a 9-day embryo in which the oculomotor nuclei lay at the rostral tip of the brain stump ascending fibers of the medial longitudinal fascicle reached the end of the brain and terminated in a small bud of brain substance. There the oculomotor nerves made their exit and mingled with the trochlear nerves just ventral to the brain.

Gaps illustrated in figures 2 and 3 were produced in the brains of nine embryos. Emerging central nerve fibers were observed in all these specimens. The facial-acoustic region was removed in two embryos which were incubated to 5 and 5½ days respectively. In both of these, descending fibers emerged from the upper stump. In the 5-day specimen the emerging fibers which apparently represented the descending component of the medial longitudinal fascicle turned forward abruptly and traveled in the pia mater for a short distance (0.4 mm.). Descending trigeminal spinal tract fibers and the descending component of the medial longitudinal fascicle emerged from the rostral stump of the 5½-day embryo shown in figure 16, turned forward in the pia mater, gradually diminished in size and ended as they reached the trigeminal level. The extramedullary course of the longest fibers of this bundle extended for approximately 0.8 mm.

A 7-day embryo had a gap from the acoustic level almost to the trochlear nucleus. The only escaping central nerve fibers were some from the lower part of the brain. These were of the type already described (pages 273 and 274) which escaped from the floor plate in a tongue of brain tissue. No central nerve fibers emerged from the upper stump of the brain of this embryo but tracts arising in this portion of the central nervous system did pursue abnormal courses in the brain wall at the site of the operation, as described on page 272.

A 9-day embryo had a gap in the upper metencephalon from the rostral trigeminal region to a point some distance caudal to the trochlear nucleus. Although complete separation of the parts of the brain occurred not much tissue was removed. From the trochlear level to the gap the brain was badly distorted and it was difficult to identify fiber tracts. Among the maze of nerve fibers were many scattered longitudinal bundles. Most of these escaped from the brain stump laterally and caudally to end in the mesenchyme. One fascicle, which appeared to be composed of descending tectal fibers, emerged ventrolaterally and then ran dorsad parallel to the extramedullary portion of the trochlear nerve. Above the roof of the neural tube it met contralateral fibers which had emerged dorsally. There a decussation was formed in the pia mater near the trochlear nerve crossing. Just lateral to the brain stem the mesenchymal cells around the trochlear nerve were oriented parallel to the nerve. Many fiber bundles which had escaped from the mesenchyme into this area had a similar alignment. One bundle actually joined the trochlear nerve. Nerve fibers emerged from the lower stump of the brain of this embryo. One large bundle followed a blood vessel forward running parallel to the rostral brain stump but making no connection with it. The bundle eventually ended in the mesenchyme between the ectoderm and the primordium of the dura mater. At the end of the caudal brain stump the ascending medial longitudinal fascicles traversed the fourth ventricle and passed into the chorioid plexus dorsally (fig. 18). There they turned caudad in the pia mater.

In brains with gaps produced operatively most of the emerging fibers wandered at random through the mesenchyme and as a rule showed no tendency to enter the other portion of the brain, although in every instance the two stumps were very close to each other and often overlapped. In only one instance was the gap between the brain stumps bridged by the emerging fibers. This occurred in the only embryo in which the gap had been produced between the oculomotor and trochlear nerve levels. The trochlear nucleus was absent on the left side. The diencephalic component of one of the medial longitudinal fascicles descended across the mesenchyme in a bridge of brain tissue to reach and enter the lower stump just caudal to the trochlear nucleus. This is illustrated in figure 15 of this article and figure 8 of a previous one (Rhines, '43). After entering the lower stump the fascicle found its proper place lateral to the floor plate and its fibers mingled with the ascending fibers of the medial longitudinal fascicle. The diencephalic component of the medial longitudinal fascicle of the opposite side emerged from the brain but instead of descending to join the caudal stump it turned rostrad along the outer wall of the diencephalon and ended after a short course.

In an embryo of 5½ days removal of the tectum was accompanied by a deficiency in the basal part of one-half of the mesencephalon. The diencephalic component of the medial longitudinal fascicle of that side emerged from the upper part of the mesencephalon and descended through the mesenchyme for a short distance, reentering the brain near the trochlear nucleus which in this case was badly distorted. From this point, however, it regained its normal position and its fibers mingled with those ascending from lower regions in the medial longitudinal fascicle.

Relation of longitudinal tracts to developing motor nuclei

The absence of diencephalic components of the medial longitudinal fascicles had no inhibitory effect on development of the cranial motor nuclei. The cranial motor nuclei differentiated in all of the operated embryos unless their primordia had been removed along with other parts of the brain. There were several specimens with no interstitial nuclei present. In these the oculomotor nuclei as well as the other cranial motor nuclei were present. Two of these specimens were 4 days old and had no longitudinal fibers at the oculomotor level. At the trochlear level four or five ascending fibers could be observed (fig. 19). After 4½ days' incubation ascending fibers from the trigeminal region formed the medial longitudinal fascicle in the caudal part of the mesencephalon. By 5 days the descending crossed tectal tract was formed at the oculomotor level occupying the region lateral to the floor plate. In these older operated embryos, however, the oculomotor and trochlear nuclei were more advanced in development than the longitudinal tracts adjacent to the floor plate (fig. 17).

The motor nuclei of the myelencephalon and spinal cord of a 5-day embryo having no brain rostral to the glossopharyngeal level developed normally. The primordia of practically the entire nuclear mass from which the descending vestibular components of the medial longitudinal fascicle arises had been removed in this specimen and of course there were no other components of the medial longitudinal fascicles.

No relationship between medial longitudinal or other longitudinal fascicles and development of commissures was indicated in the present experiments. Below the site of operation fibers crossed the floor plate as readily as they did in normal nonoperated embryos. Indeed, it was largely this that produced a normal appearance of the myelencephalon and spinal cord in embryos with forward portions of the brain removed.

DISCUSSION

The fiber pattern which characterizes the normal brain results from the direction followed by growing central nerve fibers during development. The numerous attempts that have been made to explain the direction of nerve fiber growth have resulted in a number of unsubstantiated hypotheses. Some of these have become well established in the neurological literature. Acceptance without adequate verification has led to confusion of thought.

Results of the present study lend more support to the mechanical theory of nerve growth than to others but it is not with the intention of excluding all other possible factors that evidence emphasizing the importance of the structure of the substrate is presented. It is probable that a number of interacting factors are responsible for nerve fiber growth. Furthermore, the immediate factors influencing growth are dependent upon pre-existing conditions in the embryo. For example, the alignment of micelles in the supporting framework of the neural tube may have been induced by some previous environmental influence. A consideration of the steps necessary to produce any one condition lead one back to the most fundamental questions of development. Even though other influences such as chemical and electrical factors quite possibly come into play they should not be cited as the primary guiding forces in development until some proof of their significance is forthcoming. Varied methods with careful controls will be necessary to determine their rôles in the development of the nervous system.

Although the present results do not exclude electrodynamic factors they do indicate that the action of these factors, as claimed by Kappers ('17) in his theory of neurobiotaxis, is not in agreement with observed conditions. The reversal of the course of nerve fibers under experimental conditions would seem to be impossible if conditions postulated by neurobiotaxis prevailed. An ascending fiber which turns abruptly to take a descending course can scarcely be growing with an electric current in an electrodynamic field during both parts of its course. Nor is the change in direction of the fiber due to the withdrawal of normally existing currents because the intramedullary course of the fiber is the same as in the unoperated embryo until it reaches the site of operation.

The hypothetical current influencing nerve fiber growth is supposed by Kappers to originate from sensory stimuli. Therefore loss of rostral parts of the brain and of the special sense organs should eliminate many important currents. However, after excision of all of the forebrain, the midbrain and even a large part of the hindbrain the remaining portions of the brain developed and were comparable to the normal everywhere except immediately adjacent to the incision. Even the neuroblasts of the tectum differentiated and formed tracts of normal appearance in the absence of the eyes. The early tectum (up to 8 days) could not be distinguished from that of an unoperated embryo. After about 8 days the only difference was the absence of the external fiber layer which is derived from the optic tracts.

Some experimental studies claiming to support the theory of neurobiotaxis have been published. Burr and others (Burr and Northrop, '35; Burr and Hovland, '37 a, b) found electrical potential differences between the cephalic and caudal ends of the neural tube of chick and amphibian embryos. They did not, however, establish a relationship between bioelectric fields and growing nerve fibers. The potential differences noted might have been a result rather than a cause of development.

Such observations as the very constant abnormal nerve pattern found at rounded healed stumps of brain (figs. 4 and 5), the rather definite course of the bundles of emerging central fibers if they entered the mesenchyme near cranial nerves, and the haphazard course of the central fibers emerging into a region not occupied by cranial nerves or well organized blood vessels, point to the importance of the character and alignment of the supporting elements. Whether electrical fields influence or determine alignment of the ground substance cannot be said at present. In any event, that would seem to be a secondary factor in new growth and not a source of attraction or tropism.

Other investigators (Ingvar, '20; Peterfi and Kapel, '28; Peterfi and Williams, '33) studied the effects of a galvanic current on the growth of nerve fibers in vitro and claimed a relationship between direction of the fibers and polarity of the current. Weiss ('41) repeated these experiments and found that when electrodes were so placed that they did not produce a change in the ultra-structure of the culture medium the current had no effect on fiber direction.

Hoadley ('25) transplanted the midbrain of 48-hour chick embryos to the chorioallantoic membrane. He found that growing central tract fibers emerged from the transplant. If developing tissue (myotome, cartilage, etc.) was present near the brain fragment a large fascicle innervated the neighboring tissue. In the mesenchyme alone the outgrowing fibers were short and seemed to have no particular direction. He believed that his findings supported dynamic theories.

In several of our operated embryos aimless fibers and bundles directed toward muscles were present. The region of the mesenchyme which the emerging fibers entered after leaving the brain seemed to be a differential factor. The explanation of Weiss ('41) that two proliferating centers produce stresses on the intervening mesenchyme causing a corresponding micellar alignment is supported though not proved by our observations.

Hoadley attributed outgrowth of central fiber tracts to a defect in the limiting membrane produced by operation. This was not indicated in our material. Commissural fibers escaped from the floor plate some distance from the cut surface of the brain. Furthermore there were numerous operated embryos in which no abnormal emergence of fibers occurred.

Lewis ('07) observed emerging central fiber tracts after transplanting optic vesicles to which fragments of brain were attached. He emphasized the importance of brain injury as a causal factor in this phenomenon, believing that he had changed the orientation of neuroblasts by handling the tissue with needles. We do not doubt that injury to neural tissue is the causal factor but alteration in the orientation of nerve cells rarely accompanied emergence of central tracts in our experiments. Indeed, the cells of origin of many escaping tracts that we observed were not located in the altered region of the brain and were oriented at right angles to the escaping fibers. Examples of this are ganglion cells whose fibers entered the brain, turned longitudinally, pursued a normal course in the brain and then emerged; and ascending fibers of the medial longitudinal fascicles whose neuroblasts were located at the sulcus limitans, oriented transversely.

The concept of a progressive cephalo-caudal neurofibrillar development has received considerable attention (Mesdag, '09; Bok, '15; and many others). The theory of cephalo-caudal physiological gradients (Child, '21) gave it further impetus. The attempt to fit neurofibrillar development into this concept has resulted in an erroneous interpretation of the growth of the diencephalic component of the medial longitudinal fascicle (Angulo, '39) from which false support has been offered to the claims of Coghill ('29) concerning the development of motor activity in the embryo.

The differentiation of neuroblasts in the embryonic brain does not strictly follow a cephalo-caudal order. The earliest neuroblasts to differentiate are located in the myelencephalon (Windle and Austin, '36; Rhines and Windle, '41). Shortly afterward development at the junction of the mesencephalon and diencephalon commences. From these two foci differentiation spreads forward and backward. Fibers from early neuroblasts of the hindbrain grow either rostrad or caudad and the apparent rapid elongation of fibers is actually due to the meeting of ascending and descending tracts.

This concept is contradictory to the theory of stimulogenous fibrillation in which Bok ('15) attributed development of cranial motor nuclei to radiating currents set up by the progressive descent of the diencephalic components of the medial longitudinal fascicles. The development observed in chick embryos of the present study makes this theory untenable. There was no causal relationship between differentiation of cranial motor nuclei and the downgrowth of diencephalic components of the medial longitudinal fascicles or any other longitudinal tracts.

Detwiler ('22, '23, '36) grafted super-numerary limbs opposite the thoracic spinal cord in Amblystoma embryos. He attributed the failure of hyperplasia of primary motor nuclei to the relatively small number of fibers from the brain growing into this region as compared with the cervical cord. He stated that his work supported Bok's theory of stimulogenous fibrillation. However, Hamburger ('34) who, like Bok, used the chick embryo found hyperplasia in experiments similar to Detwiler's. Furthermore hyperplastic responses are not applicable to Bok's theory which dealt with the initiation of development of motor nuclei. As can be observed in the normal chick, cat and human embryo the first spinal motor neurons begin to develop and form ventral roots before any longitudinal tracts have formed.

Oppenheimer ('42) suggested that in Fundulus embryos the longitudinal direction taken by Mauthner's fibers after crossing in the floor plate might be due to the arrival of the descending medial longitudinal fascicle coincident with emergence of Mauthner's fibers from the floor plate. This explanation does not seem to hold true for commissural fibers in the chick; deflection of crossing fibers was produced by folds in the floor plate. Furthermore, longitudinal fibers were caused to take transverse courses by changing the shape of the brain. In very early normal embryos (3- to 4-mm. cat and 18-somite chick embryos) transverse fibers appeared in the floor plate before any longitudinal fibers were present. The first longitudinal fibers of the spinal cord and hindbrain were observed a short time later and they had their origin from commissural fibers. Recently Piatt ('44) after performing further experiments on Amblystoma embryos concluded that the decussation of Mauthner's fibers was independent of the presence of the diencephalic components of the medial longitudinal fascicles.

Numerous experiments by Harrison ('35) and Weiss ('39, '41) support the mechanical theory. Many of these were studies in vitro and most of them were concerned with growth of peripheral nerves. Our experiments were limited in scope and did not afford an opportunity to test all factors but the results obtained do suggest the importance of the ground substance in influencing direction of growing fibers in the central nervous system. There was a very constant correlation between alteration in the shape of the neural tube and change in the fiber pattern. The polarity of neuroblasts is influenced by the alignment of the substrate as evidenced by the radial arrangement of neuroblasts at the rounded stumps of operated brains. Longitudinal fiber tracts follow the contour of the supporting framework. That the arching and decussation of fiber tracts at dome-shaped stumps of the brain was not merely due to loss of an attractive force was indicated by the fact that when there were slight irregularities in the shape of the stump longitudinal fibers became diverted from their arching course and emerged from the brain.

The course of emerging central fiber tracts did not indicate the presence of any tropic influence. The direction of such fibers appeared to be aimless and conformed to the alignment of the mesenchyme and ectoderm with which they came in contact unless they emerged into the mesenchyme in which blood vessels or cranial nerves were pursuing definite courses. In the latter instances they ran parallel to these structures in the mesenchyme.

In brains separated by gaps the failure, except in one case, of emerging fiber bundles to connect with the separated segment points to the fact that structural entities, such as mesenchyme and pia mater, play important roles in orienting the fibers. We saw no evidence of attraction exerted by growing nerve centers. Comment on the very definite connections established between the separated rostral and caudal parts of the midbrain in the one exceptional case must be reserved until more specimens can be operated upon at this level. The connecting fascicle ran in a thin strand of neural tissue. The separation of the parts at operation may have been incomplete.

Nicholas ('29, '30) produced gaps in the hindbrain of Amblystoma embryos. He prevented union of the stumps by transplanting a pronephric tubule or a limb into the gap. Though his experiments are not comparable with ours, he did observe that central tracts emerged from the caudal stump and made functional connections with the transplanted limb. Jones ('37), Fugo ('40), Stultz ('41) and Bueker ('43) have experimented with removing parts of the nervous system but none of them has published results on growth of fibers after operations. This method of experimental embryology when combined with silver staining is one deserving of more extensive use.

SUMMARY

The effect of surgical removal of portions of the brain upon development and course of central fiber tracts was studied in chick embryos. Operations were performed at 38–40 hours' incubation, a stage at which neurofibrillar development had just begun and at which no longitudinal fiber tracts were present in the embryo. In some embryos the forebrain or the forebrain and midbrain or the forebrain, midbrain and a large part of the hindbrain were removed. In others a segment of the neural tube was excised at the mesencephalic or metencephalic levels. After

operation the embryos were allowed to continue incubation up to 4 to 9 days. They were subsequently prepared for histologic study by the pyridine-silver technique.

The cut surface of the brain usually healed to form a dome-shaped stump in which the nerve fiber pattern was altered to conform with the contour of the supporting framework. Longitudinal tracts arched across the healed end of the brain forming decussations in most instances. In two embryos they curved toward the midline, resumed their original direction and emerged from the stump. Axons of neuroblasts located in the stump formed a radial or wheel-shaped commissure from which fibers entered the longitudinal tracts of the basal plate. In specimens with the myelencephalon isolated from the metencephalon the healed end appeared rather primitive and, although there were many fibers present, most tracts could not be identified. In the rostral segment of two brains with gaps through the upper metencephalon the descending diencephalic components of the medial longitudinal fascicles formed a partial decussation caudal to the trochlear nucleus. In the metencephalon of two embryos fibers which normally would form commissures were diverted to a longitudinal course in the midline near a pocket in the floor plate.

In addition to alterations in central course of fibers some tracts emerged from the brain to take up a peripheral course. Most of them merely wandered in the mesenchyme sometimes reaching the neighboring ectoderm before ending. Fibers that left the brain near the exit of the oculomotor nerves formed a bundle which followed a course parallel to the oculomotor nerves. In some specimens such fibers were applied to an artery wall during their peripheral course. Frequently commissural fibers at the abducens level formed a thick bundle which left the midline of the floor plate to end near the notochord. In several instances fiber tracts reversed their course after emerging.

In all except one embryo gap in the brain failed to become bridged by emerging fibers even though the separated parts of the brain lay very close to one another.

No relationship could be observed between the presence of the diencephalic component of the medial longitudinal fascicle or any other longitudinal tract and the longitudinal course taken by commissural fibers after crossing the midline. Development of cranial motor nerve nuclei was observed in the absence of all longitudinal tracts. The present experiments would seem to lend no support to the theories of neurobiotaxis and stimulogenous fibrillation. Although the nature of experiments made it impossible to exclude all factors results obtained seemed to indicate that the orientation and structure of the substrate was an important factor in directing the growth of new nerve fibers in the central nervous system.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

All illustrations are unretouched photomicrographs of pyridine-silver stained preparations of chick embryos operated upon at 38–40 hours of incubation. Magnification of all figures is 100 ×.

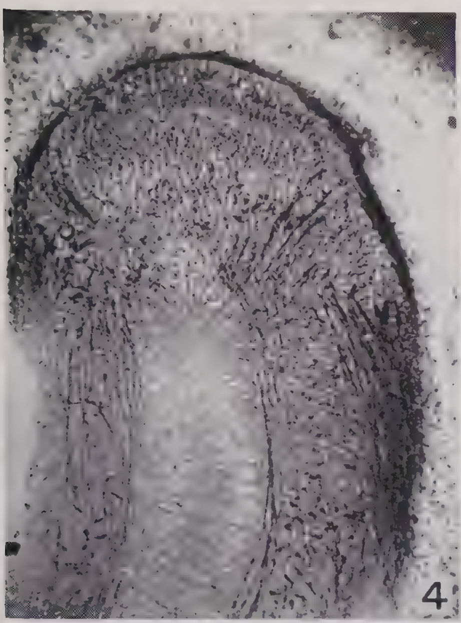
4 A frontal section through the rostral end of the brain stump of a 5-day embryo, no. 8A, which was operated upon at the level indicated in figure 1, D. Due to ventral flexure at the tip of the stump the ascending primary afferent tracts, forming an arch at the healed end of the brain, appear in the same section with the more caudal floor plate. Nerve fibers from neuroblasts in the reticular formation form a radial commissure in the rostral end of the floor plate. A little more caudally the ventral commissure assumes its normal pattern. The ventral longitudinal fascicles flank the floor plate and bundles from the medial longitudinal fascicles curve toward the midline.

5 A frontal section through the healed stump of a 6-day embryo, no. 5 b, operated upon at the level indicated in figure 1, D. It is slightly more dorsal than the section in figure 4. Ventral flexion of the tip of the brain brings the ventricle and the roof plate into the same section as the floor plate. Around the rostral end of the floor plate, containing a wheel-shaped commissure, the medial longitudinal fascicles form a decussation. Some fiber bundles continue rostrad and end within the brain.

6 A 5½-day embryo, no. 2N, which was operated upon at a level indicated in figure 1, D. This illustrates a transverse section through the caudal part of the trigeminal region of the brain. The much thickened floor plate contains a wheel-shaped commissure and the medial longitudinal fascicles are crossing at the place indicated by the arrow.

7 A 7-day embryo, no. 2v, operated upon at the level indicated in figure 3. This illustrates a frontal section a short distance from the end of the caudal stump. Commissural fibers are turning rostrad into the floor plate where they form a thick bundle between the two medial longitudinal fascicles.

8 A 9-day embryo, no. 4v, operated upon at the level indicated in figure 2 B. This illustrates a frontal section through the basal portion of the mesencephalon. The oculomotor nuclei appear in the upper third of the illustration. Due to the obliquity of the section only the left trochlear nucleus appears and this is the darker mass on the left of the middle third of the picture. The diencephalic components of the medial longitudinal fascicles form a partial commissure caudal to the trochlear nucleus. This is indicated at the upper arrow. Many of the fibers continue caudad in bundles near the midline as indicated by the lower arrow.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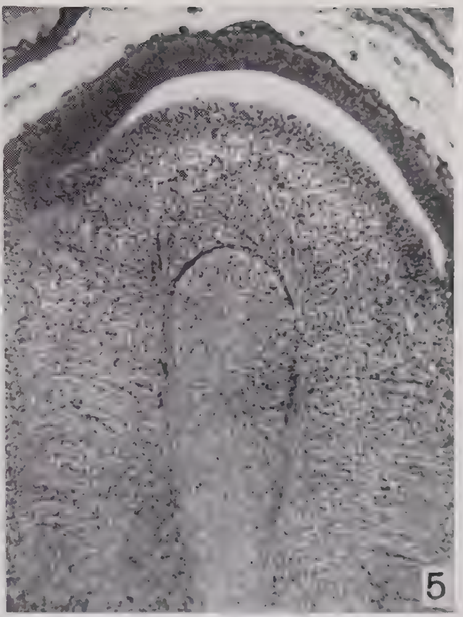
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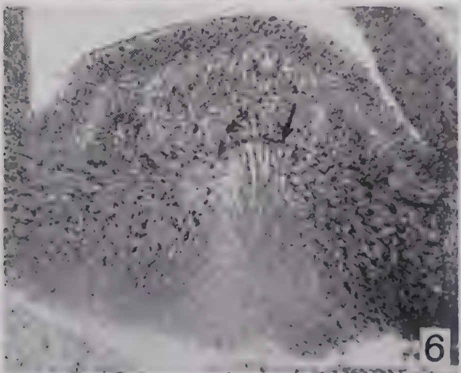
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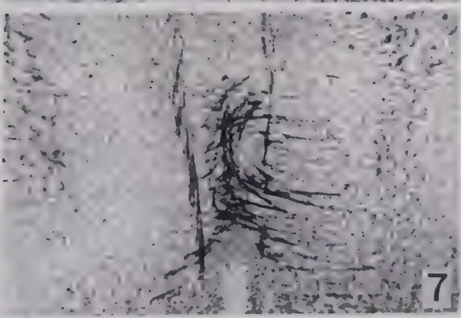
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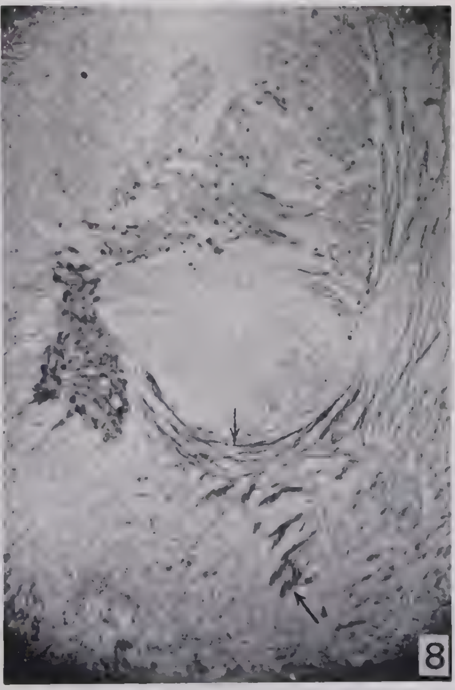
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PLATE 2

EXPLANATION OF FIGURES

All illustrations are unretouched photomicrographs of pyridine-silver stained preparations of chick embryos operated upon at 38–40 hours of incubation. Magnification of all figures is 100 ×.

9 A 4½-day embryo, no. 14B, which was operated upon at the level indicated in figure 1, D. This illustrates a transverse section through the brain at the trigeminal level where nearly normal structure prevails. Longitudinal nerve fibers from neuroblasts in the healed stump a little rostral to the level shown have formed a tract which is seen here in the floor plate indicated by the arrow.

10 A 4½-day embryo, no. 11A, operated upon at the level indicated in figure 1, D. This illustrates a transverse section near the healed end of the brain. Part of the sensory root of the trigeminal nerve appears on the extreme left. Prominent bundles of nerve fibers from local neuroblasts in the brain wall emerge ventrally into the mesenchyme. The resemblance to peripheral nerves is striking.

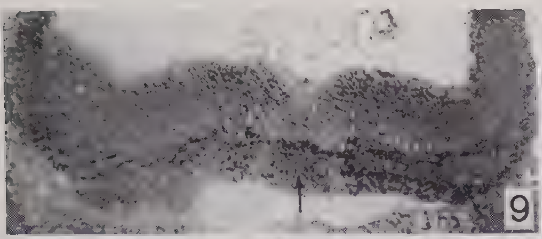
11 A 6-day embryo, no. 6Z, which was operated upon at a level indicated by figure 1, D. This is an oblique frontal section through the healed stump of the brain at the trigeminal level. On the right side a few sensory root fibers from the trigeminal ganglion may be seen entering the brain. The right medial longitudinal fascicle emerges from the rostral end of the brain with ascending primary afferent fibers and fibers of the reticular formation. Similar fibers from the left side, not shown in the photograph, also join the emerging bundle. A cyst of pia mater is located at the end of the brain to the right of the emerging bundle. The emerging bundle runs to the ectoderm beneath which it ends.

12 A 5½-day embryo, no. 8L, operated upon at the level indicated in figure 1, A. The photograph shows a region in the mesenchyme ventral to the cephalic flexure which in this instance lies between the healed rostral stump of the brain and the upper part of the metencephalon. Between the two prominent oculomotor nerve bundles lies the unpaired internal carotid artery. To the right of this artery is an oculomotor rootlet which has failed to join the main trunk. Rostral to the artery and at the top of the illustration is a bundle of nerve fibers which has emerged from the stump of the caudal part of the diencephalon. At a level more caudal than that shown here this bundle applies itself to the wall of the internal carotid artery.

It will be noticed that the oculomotor nerves are unusually large in this specimen. Apparently they contain fibers ascending from sources other than the oculomotor nuclei.

13 This is the same embryo as in the preceeding figure. The section is through the rostral stump of the brain. Part of the tectum attaching to the basal portion of the brain is shown in the upper right. The right oculomotor rootlets appear in the center at the bottom of the photograph. Ascending nerve fibers arch over the end of the brain; many of them emerge on the right side forming large bundles at the arrows. This is not the source of the bundle shown in figure 12.

14 A 5-day embryo, no. 5 O, which was operated upon at a level indicated in figure 1,E . The section passes transversally through the glossopharyngeal region. A bundle of commissural fibers emerges from the floor plate and passes for a short distance into the mesenchyme like a peripheral nerve. Almost the entire extent of this bundle is illustrated here. For purposes of orientation, the glossopharyngeal nerve appears on the extreme left of the illustration. The otocyst appears in the lower right hand corner under the figure number.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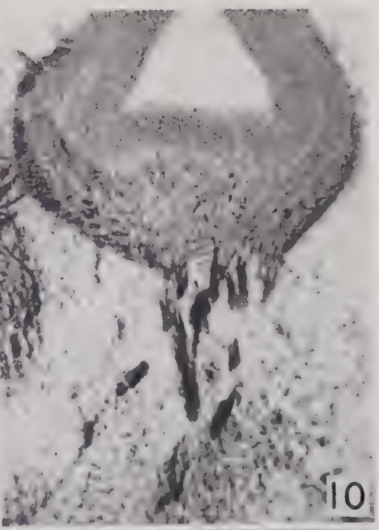
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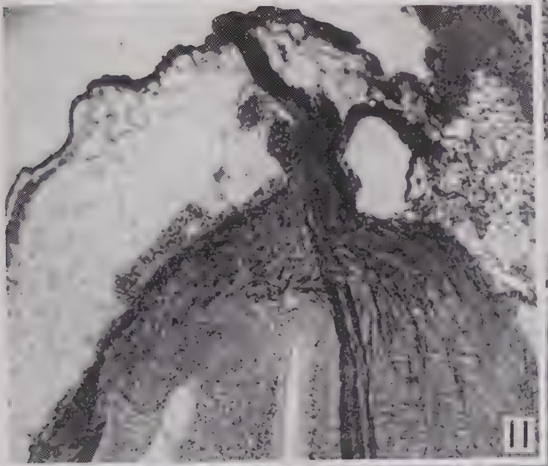
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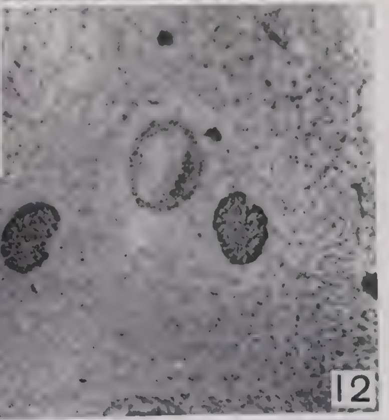
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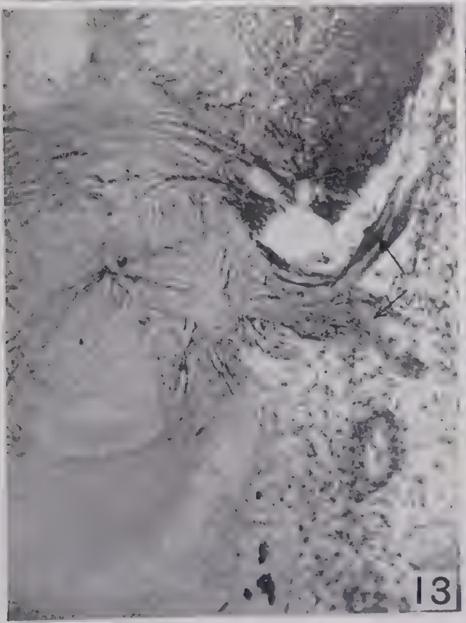
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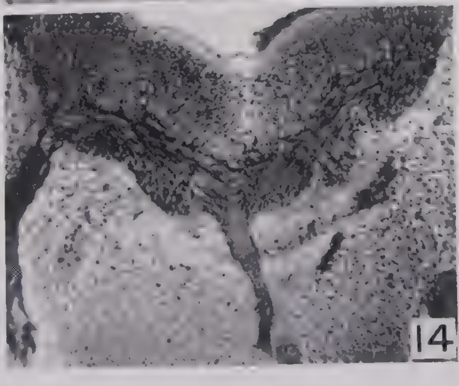
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PLATE 3

EXPLANATION OF FIGURES

All illustrations are unretouched photomicrographs of pyridine-silver stained preparations of chick embryos operated upon at 38–40 hours of incubation. Magnification of all figures is 100 ×.

15 A 5½-day embryo, no. 5N, which was operated upon at the level indicated in figure 2, A. The caudal stump of the diencephalon and upper mesencephalon is shown in the upper center of the picture. A bundle of central fibers accompanied by a strand of brain tissue is shown entering the caudal segment of the brain near the trochlear nucleus. This is the diencephalic component of the medial longitudinal fascicle of one side. It has crossed its point of origin in the rostral segment of the brain. This illustration is about 120 microns from figure 8 of a preceeding paper (Rhines, '43).

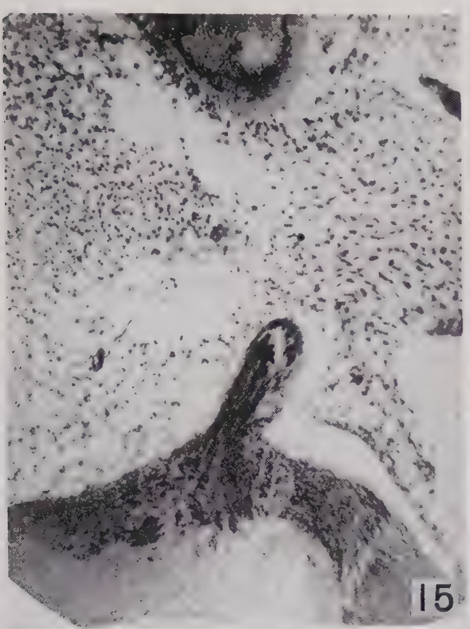
16 A 5½-day embryo, no. 6N, which was operated upon at the level indicated in figure 2, C. This illustrates a transverse section through the caudal end of the rostral brain segment in the lower trigeminal region. The darkly stained bundles of nerve fibers coursing in the pia mater ventral to the brain (in the wall of the shrinkage space) have emerged from the brain more caudally. They belong to the medial longitudinal fascicle and to the spinal tract of the trigeminal nerve. These fibers are actually ascending on the outside of the brain, having turned rostrad after emerging from the stump of the brain.

17 A 5-day embryo, no. 15B, which was operated upon at the level indicated in figure 1, B. This illustrates an oblique transverse section with the trochlear nucleus visible on the left side. The right side is caudal to the left. The medial longitudinal fascicle is absent on the left side. Fibers seen ventral to the trochlear nucleus belong to the crossed tectal tract.

18 A 9-day embryo, no. 4v, which was operated upon at the level indicated in figure 2. B. This illustrates the dorsal part of the caudal stump in the trigeminal region. The ascending medial longitudinal fascicles have passed dorsad and are shown emerging from the brain into the pia mater before reversing their course. They are indicated at the arrows.

19 A 4-day embryo, no. 11C, which was operated upon at the level indicated in figure 1, B. The section passes through the cephalic flexure at the stump of the brain. The lower two-thirds of the photograph contains the mesencephalon in oblique transverse section. The trochlear nuclei appear to be normally developed although no diencephalic components of the medial longitudinal fascicles are present. There are a few (five or six) ascending fibers of the medial longitudinal fascicle on either side, scarcely visible in the photograph.

At the top of the illustration are two crescent-shaped masses, one on either side of the brain stump. These are the oculomotor nuclei.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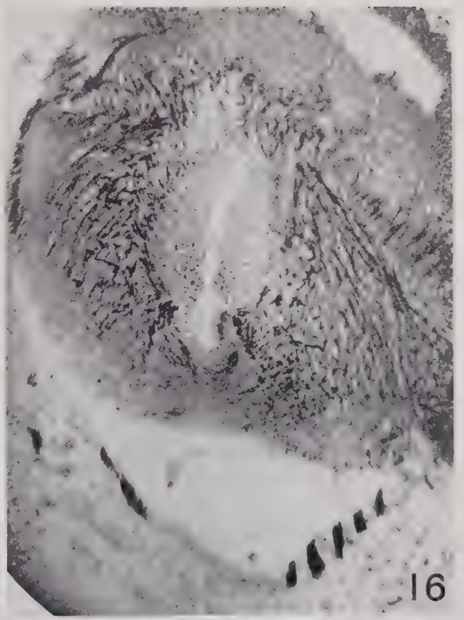
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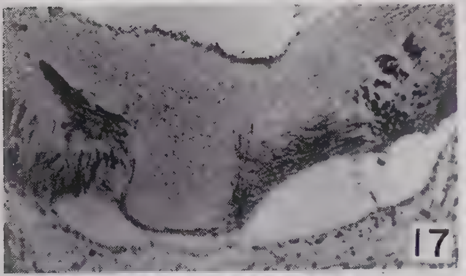
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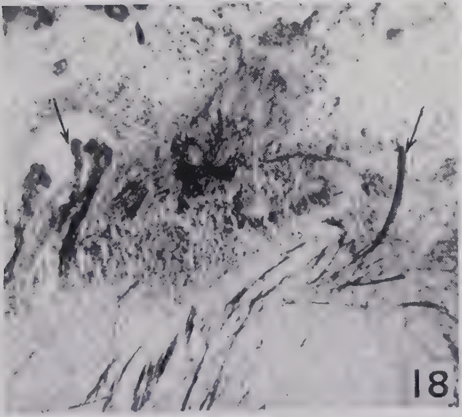
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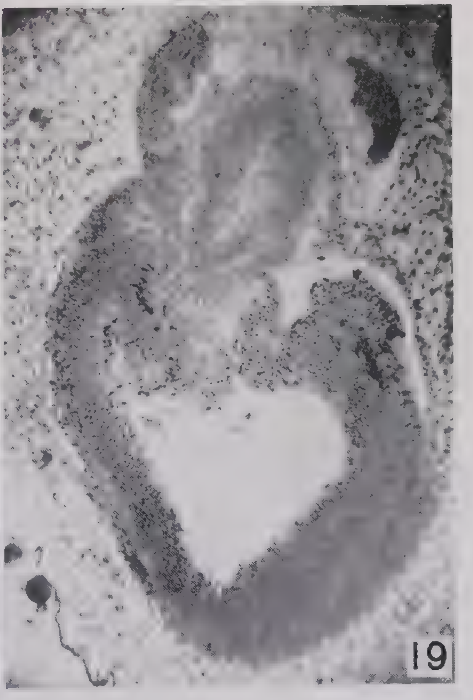
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CHANGES IN MEDULLARY BONE DURING THE REPRODUCTIVE CYCLE OF FEMALE PIGEONS

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ONE FIGURE

It was shown by Kyes and Potter ('34) that some female pigeons have solid leg bones, while males uniformly have marrow-filled bones. They further stated that the osseous changes in females must be cyclic, since higher degrees of ossification were associated with ovarian follicles of larger size. Macroscopic and histological observations were made on the nature of this bone, but Kyes and Potter did not state that the females showing one or another degree of formation of medullary bone were "virgin" females — i.e., females close to the ovulation of the first egg in life. Yet, as shown by data presented below, it is only in such "virgins" and in carefully selected laying birds, that a significant correlation between follicle size and degree of ossification can be found. Pfeiffer and Gardner ('38) induced the formation of medullary bone in both male and non-laying female pigeons by injections of estrogen, and published figures showing clearly the gross changes in the femurs of treated pigeons and also of an untreated female at normal egg-laying. Bloom, Bloom and McLean ('41) studied the histology of medullary bone formation and resorption in the femurs of twelve females at or near ovulation, including shell-formation, and of one bird at 6 and another at 10 days after laying. Only data from the two last-named cases bear on the question whether and how soon medullary bone is completely resorbed. The literature provides only inadequate evidence that most or all of the medullary bone formed at one preovulation stage is resorbed before the onset of a next following preovulation period. Thus, the extent to which medullary bone formation and resorption is truly cyclic in the pigeon is inadequately known, and the factors affecting the resorption of medullary bone are almost entirely unexplored. The purpose of the present study was to obtain information on these questions.

MATERIAL

Nearly all of the 126 female pigeons used in this study were bred in our own colony, and for these birds complete records for age and egg-laying were available. A few adults were purchased; for these birds only year of hatching and date of laying of terminal clutches of eggs were known. Birds varying in body size and breed, mostly crosses involving two or three breeds, were used. Differences in body size are relatively unimportant in these tests since they apparently do not affect rate of bone formation or resorption, and the associated differences in size of follicles (ova) and oviducts are small in comparison with differences due to stage of reproduction. Some excess of mixed grain (cracked yellow corn, peas, Kaffir corn and wheat), water, and mixed "grit" — each in separate containers — were provided at all times. The commercial grit contained oyster shell, calcite, sand, salt, charcoal and Venetian Red. Most birds were exposed to direct sunlight while maturing and also later except during cold weather. Some heat was provided in glass-sided houses in winter. Birds were sacrificed and studied at all seasons.

Birds which had previously produced eggs (adult) and those which had not (virgin) were killed at known stages of the reproductive cycle; other virgin birds (aged 5.0 $^+$ months) were killed in order to learn the precise state of the reproductive organs and of femur ossification. Some birds killed at 3 to 22 days after laying were found (femurs ossified) with a new crop of ova 6 to 20 mm. in diameter; data from birds of this type are excluded from table 1, since other data demonstrate that in these cases two crops of ova had opportunity to share in the observed state of ossification of their femurs. For purposes of this study a bird that had laid but a single pair (clutch) of eggs, when killed before the onset of growth of a second pair of eggs in the ovary, is still called "virgin"; this is necessary, since the resorption of the first medullary bone formed in association with growth of the first pair of follicles is a part of a single — and first — cycle.

Only the femurs were studied. The shafts of femurs of freshly killed birds were first broken near the middle. When superossification was present the broken ends were sliced obliquely with a sharp scalpel, and studied macroscopically. In the case of marrow-filled shafts the endosteal borders were explored with a dissecting needle or fine-pointed forceps; in this way grades zero and 1 could be distinguished. Grades 2 to 4 were readily determined by the extent to which medullary bone had displaced the marrow in the cross-section of the femur. Femurs of grade 4 were either completely ossified or they showed only a very fine line of unossified marrow in or near the center of the cross-section.

Determinations of total plasma calcium were made on pigeons of various breeds at all stages of the reproductive cycle; in many cases the same female was measured at three or four stages of the cycle. The data provide information concerning the period of increase and decrease of estrogen production in the pigeon, in addition to showing the type or direction of blood calcium change which accompanies both the formation and resorption of endosteal bone. More than 90% of the calcium determinations were made on plasma by the method of Clark and Collip ('25). A few determinations were made colorimetrically.

RESULTS

Birds examined in the earliest stages of maturity and of egg formation are grouped at the top of table 1, and progressively later stages follow in their true order. The position or relative order of birds examined in the earliest reproductive stages (resting and preovulation)

TABLE 1

Grade of femur ossification in female pigeons at the various stages of the reproductive cycle.

VIRGIN OR ADULT PIGEON	STAGE OF REPRODUCTIVE CYCLE	DIAMETER OF LARGEST OVUM	AVERAGE WEIGHT OF OVIDUCT	GRADES OF OSSIFICATION AND (BELOW) NUMBER OF CASES					HARD OR SPONGY BONE
				0	1	2	3	4	
		mm.	*gm.*						
Virgin	Resting	<1.0	<0.10	26	..	..	..	..	
Virgin	Resting	1.5–2.8	0.16	8	1	..	..	..	
Virgin	Resting (?)	3.0–4.1	0.91	1	2	3	1	..	Hard [1]
Virgin	Preovulation	7.3	2.90	..	..	1	..	..	
Virgin	Preovulation	14.2–17.0	8.23	..	..	..	3	2	Hard
Adult	Preovulation	5.8–9.7	1.65	..	..	1	4	3	Hard
Adult	Preovulation	12.7–18.5	8.18	..	..	..	..	3	Hard
Virgin	Soft shell (1st egg)	18.0	8.68	..	..	..	..	2	Mixed
Adult	Soft shell (1st egg)	17.2	8.55	..	..	..	..	6	Hard
Virgin	Hard shell (1st egg)	18.5	9.42	..	..	..	..	2	Mixed
Adult	Hard shell (1st egg)	19.7	9.00	..	..	..	2	4	Mixed
Adult	Soft shell (2nd egg)	(6.0)	9.33	..	..	..	..	6	Hard
Adult	Hard shell (2nd egg)	(5.0)	8.50	..	..	..	..	5	Spongy
Virgin	2– 6 days after egg	4.4	1.51	..	..	..	2	1	Mixed
Adult	2– 6 days after egg	5.2	2.16	..	..	..	1	6	Mixed
Adult	9–14 days after egg	4.2	1.05	..	..	2	2	4	Mixed
Adult	20–23 days after egg	3.8	0.98	3	...	1	2	2	Spongy
Adult	25–28 days after egg	3.6	0.73	1	1	1	2	3	Mixed
Adult	37–60 days after egg	2.8–4.8	0.95	1	...	1	3	..	Spongy
Adult	54–81 days after egg [2]	2.2	0.28	2	..	..	..	1	Spongy
Adult	14 days (parathydect.)	2.7	1.62	..	..	..	..	4	Mixed

[1] Applies only to ossification of grades 3 and 4.

[2] On a deficient mineral diet (grain and crushed oyster shell only) for 66 days.

was determined solely on the basis of diameter of the largest ovum in their ovary. The data show that low grades of ossification of the femur usually occurred when the ovarian follicles (ova) attained a diameter of 2.8 to 4.0 mm., and that this was accompanied by a very marked increase in the weight of the oviduct; the latter fact indicates an increased production of estrogen by the bird. Complete ossification usually occurred when, or soon after, the follicles attained a diameter of 9 to 10 mm. This superossification consists of hard bone of pale pink or chalky color. In femurs of birds killed while shell was being deposited (on either the first or the second egg) the endosteal bone was usually reddish and spongy, indicating some resorption of the hard bone that was uniformly present at earlier stages. In thirteen of fourteen birds killed with soft-shelled eggs in their oviducts the endosteal bone was hard and lightly colored.

The data show that after eggs are laid some resorption of the new bone occurred in many individuals, but that complete resorption was not observed in a period shorter than 22 days; also that little or no resorption — beyond that accomplished during the formation of the shell of the second egg — occurred over a still longer period in the case of several females. Three birds were subjected to a diet especially deficient in sodium chloride for 66 days, and examined at 54 to 81 days after their last egg; the rate of bone resorption was not clearly affected by this treatment.

Five pigeons were parathyroidectomized on or near the day they completed the laying of a clutch of eggs. They were given a normal diet plus 0.2 gm. daily of calcium in the form of calcium gluconate, and injected daily with 0.1 ml. dihydrotachysterol (AT10); despite this treatment two birds twice exhibited tetany. One of this group of birds was suspected of laying an egg at 5 days after parathyroid removal and was killed at 9 days. The femur of this bird was completely ossified. The other four birds were killed at 14 days after laying and parathyroidectomy. The data of table 1 show that the femurs of all these birds had ossification of grade 4, although spongy bone was present in three cases. The number of these operated birds is too small to be decisive, but 50% of unoperated birds killed at 9 to 14 days after laying showed ossification of a grade less than grade 4. The slight evidence obtained from this group of birds suggests that bone resorption proceeded less rapidly in the parathyroidectomized birds. These birds, however, were being given an excess of a soluble calcium salt, and also treated with a substitute (AT10) for parathyroid hormone; it is possible therefore that this treatment, rather than the absence of the parathyroids, was responsible for absence of bone resorption in these cases. At the end of their test the ultrafiltrable calcium of these five birds was only slightly below normal (average, 5.9; range, 3.6 to 6.5 mg. per cent).

Information concerning the amount of time involved in the superossification of the femur, and in the ovulation and formation of the two eggs, is available. Various studies have indicated that endosteal bone formation, increased weight of oviduct, and increase of plasma calcium, all result from a temporary increase of estrogenic hormone in the blood; probably the three processes begin simultaneously or nearly so. Data were earlier reported for cyclic changes in weight of the oviduct (Riddle, '27) and for blood calcium (Riddle and Reinhart, '26) in ring doves. All stages of the reproductive cycle of pigeons now appear to be measurably longer than those of doves. It therefore seems useful to provide information obtained exclusively from pigeons. For this purpose the changes in plasma calcium, referred to known points on a time scale, are presented in figure 1. From that curve it is evident that at 131 hours before an ovarian egg is to be ovulated the plasma calcium is already markedly increased; and it is wholly probable that medullary bone formation is initiated at or before that time. This is partly confirmed by the observation (table 1) that ossification may begin in virgin females when their ova are not more than 3.0 mm. in diameter, and by other unpublished observations which indicate that approximately 108 hours are required for ova of 5.0 mm. to mature and ovulate. The time required for ova of 3.0 mm. to attain a diameter of 5.0 mm. is unknown. The peak of the blood calcium curve is reached at about 35 hours before the ovulation of the first ovum. Grade 4 ossification of femurs is usually completed earlier, probably at 50 to 70 hours before that ovulation.

Concerning the data presented in figure 1 it should be noted that part of the marked variability of the plasma calcium values resulted from the use of several distinct breeds of pigeons. Although the various breeds show practically the same level of blood calcium during reproductive rest, the heavy breeds (Carneau, King, Runt, Homer) showed greater increases during ovarian activity. Although the resulting data are not included in the curve the blood calcium was measured in birds near the end of the incubation period and at or before the eighth day of feeding young (26 days after laying eggs). The values obtained did not differ significantly from those obtained during early incubation and the "resting" period.

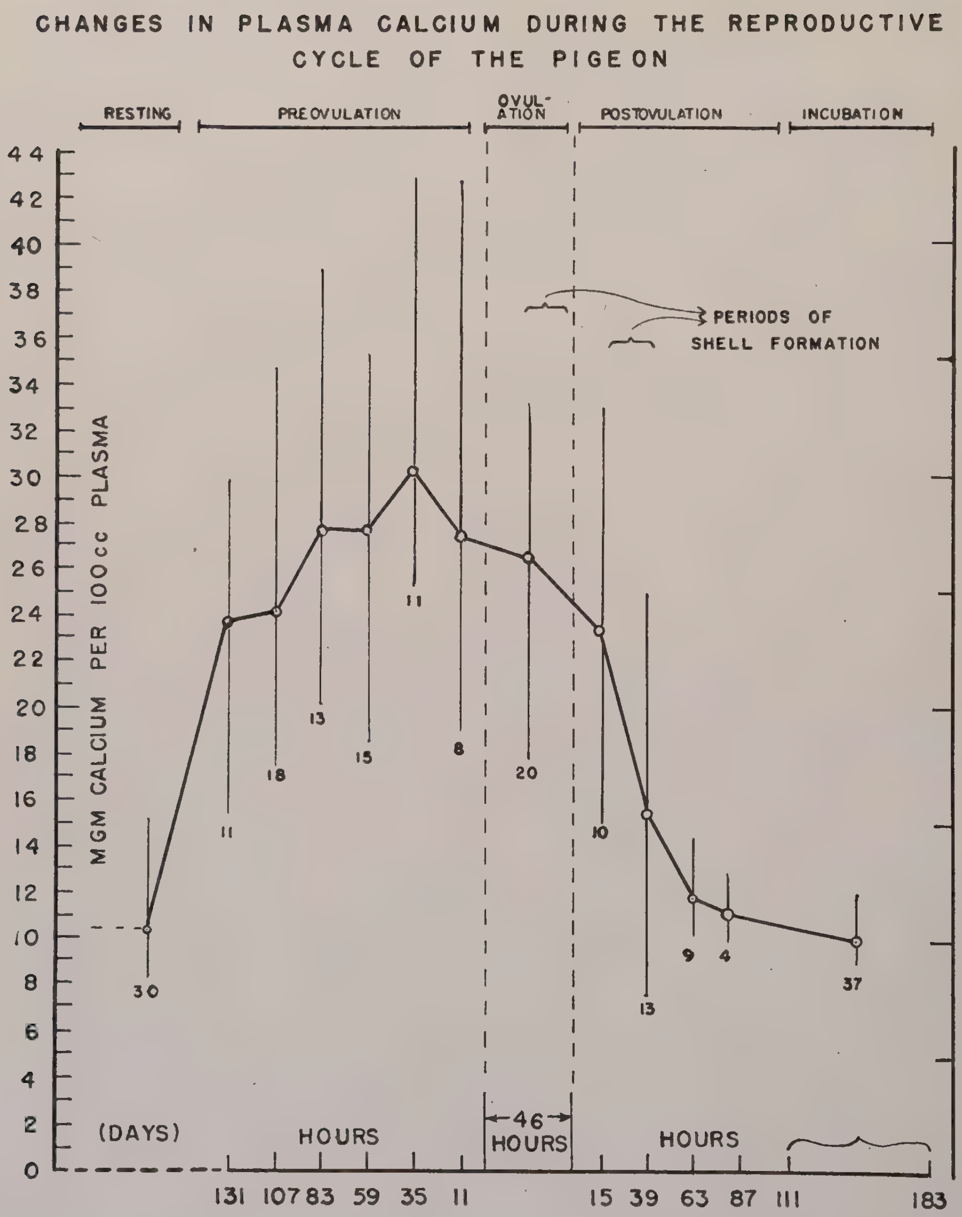

Fig. 1 Changes in plasma calcium of female pigeons at and near egg production, with indication of the amount of time included in some stages of the reproductive cycle. The vertical lines show the range of calcium values obtained at each period. The points joined to form a curve represent averages of the several determinations. Numbers refer to the sum of plasma calcium determinations.

DISCUSSION

Femurs were found to be free of visible medullary bone in some female pigeons whose egg-laying records, ovaries and oviducts gave evidence of ovarian inactivity for 22 or more days. Femurs of some additional birds showed ossification of grade 2 as soon as 9 to 14 days after egg-laying, although an equally low grade of ossification was not obtained among nine birds observed after 2 to 6 days. Birds of the above types therefore provide proof that complete and nearly complete resorption of medullary bone occurs cyclically in some pigeons. However, two-thirds of the birds subjected to this test retained for 20 to 28 days either a reduced amount of medullary bone or the large amount and type of bone which was prevalent in birds killed while shells were forming on second eggs. Since many or most of the pigeons of this colony were known to start the growth of a new crop of ova at 25 to 30 days after laying, it can be concluded that only partial resorption of medullary bone occurred between their successive egg cycles.

Three things are notable concerning those birds which maintained femur ossification of grades 3 or 4 for a period of 20 to 60 days. First, their number is greater than that of birds which attained grade zero during this period. Second, some resorption of bone had occurred even in these femurs, since spongy bone (red, suggesting increased blood supply) had apparently supplanted the hard bone of preovulation stages in all except three cases. In these three birds the diameter of the largest ovum and the oviducal weight exceeded the averages for groups to which these birds belonged; it therefore seems more probable that a very early phase of a new ossification had changed spongy to hard bone than that no resorption of bone had followed egg-laying in these cases. Third, in birds killed one or two months after normal ovulations which resulted in egg-laying there is a possibility that a later and abnormal ovulation (to body cavity) produced new bone formation nearer to the time the bird was killed. Concerning the complete resorption of medullary bone, Bloom, Bloom and McLean ('41) noted only that very little of it was present in one bird killed at 6 days after laying, while in another bird killed 10 days after laying ''the marrow had returned practically to that of the pigeon in the resting stage''. It has been noted that the latter stage was never attained sooner than 22 days after laying in the present tests. Comparison of the data obtained in the two laboratories clearly indicates that hyperossification was more rapid (probably also more complete), and bone resorption was much less rapid, in the birds studied in this laboratory.

Bloom, Bloom and McLean ('41) described rapid and extreme reversals in the cellular activity which accompanies the deposition and resorption of medullary bone within the short period between the laying of the two eggs. They state (p. 456): "Thus in the 40 hours which elapse between the laying of the first and second eggs, the bone marrow undergoes two extreme reversals in cellular activity. The period begins with a high grade bone destruction which lasts for probably 4 or 5 hours and is a continuation of the previous period of bone resorption. This gives way abruptly to a period of most intense bone formation which lasts for perhaps 20 hours and is in turn followed by about 10 hours of intense bone destruction." These changes are likewise indicated in table 1 by changes in texture of medullary bone, from hard to spongy or the reverse. The period of bone destruction during the formation of shell on the second egg coincided with a change of hard bone to spongy bone in all of five tests. At the corresponding period in the deposition of hard shell on first eggs this change of hard to spongy bone apparently occurred in four of the six (grade 4) females.

In their histological studies Bloom, Bloom and McLean ('41) seem not to have encountered a case in which hard bone completely obliterated the marrow cavity. They state (p. 465): "The transitory bone formation connected with ovulation takes place in the granulocytopoietic portions of the marrow. The blood vessels are apparently not encroached upon. Erythropoiesis is not greatly affected during the formation of medullary bone although myelocytopoiesis practically disappears from the marrow at this time." When we obtained maximum ossification (hard whitish bone of grade 4 with no central fine line of unossified marrow) it seems nearly certain that all larger blood vessels had disappeared from the marrow area. Indeed, our experience with these naturally occurring cases, together with success in the induction of similarly complete ossification of the femurs of pigeons with estrogens (Riddle, Rauch and Smith, '45), leads us to suggest that clinical importance may attach to this ability of estrogen to suppress and destroy most marrow cells. If similarly complete ossification of the marrow of human bones is obtainable with estrogen it may prove practicable to combat some of the rare and fatal blood diseases by temporary hyperossification with this hormone.

The observations which associate resorption of endosteal bone with the period of shell deposition provide no information concerning the mechanism of this phase or degree of bone resorption. A special application of the factors considered in the following paragraph suggests that two periods of markedly reduced estrogen production in the bird's ovary may provide the reason for the two periods of bone resorption which happen to coincide with a part, and apparently with only a part (10 of 25 hours), of the period of shell formation. If estrogen is secreted chiefly in cells of the two growing follicles, but only prior to their collapse (at periods separated by 46 hours) and beginning atrophy, two sudden reductions in the supply of circulating estrogen would thus result. Actual decrease of estrogen at or before the period of shell formation on second eggs was clearly indicated by a decrease of oviducal weights of ring doves at that period (Riddle, '27). The amount of time which must elapse between a diminution of the estrogen supply and the onset of bone resorption can not be stated merely in terms of hours, but it is known that the one follows the other. Recalcification or hardening of spongy medullary bone during one or another interval (20 to 25 hours according to Bloom et al.) following the bursting of the second follicle could be interpreted as a result of increased amounts of estrogen produced during the last day of growth in this rapidly expanding follicle.

In doves and pigeons it is practically certain that temporary increase and decrease of oviducal weight are the result of increase and decrease, respectively, of estrogen in the blood (Riddle, '42; Lahr and Riddle, '44). The tabulated data show that complete bone resorption, following egg production, occurred only when the oviduct had undergone rather extreme reduction in weight; and it is known that estrogen induces the formation of endosteal bone (Zondek, '37; Pfeiffer and Gardner, '38) even in the absence of the parathyroids (Riddle, Rauch and Smith, '45). Therefore the resorption of endosteal bone, like the atrophy of the oviduct, is probably partly caused by the diminution or disappearance of estrogen from the bird's blood; and it is evident that resorption of endosteal bone can be completed only when this diminution of the supply of estrogen is sufficiently prolonged. The following observations suggest, however, that the disappearance of estrogen from the blood is not the only hormonal factor essential to the resorption of endosteal bone: (a) In the present tests two instances of extreme atrophy of the oviduct were not accompanied by complete resorption of bone. (b) Pigeons in two different laboratories have given evidence of very unequal rates of bone resorption; differences in diet and exposure to light, acting either directly or through the parathyroids, might account for these exceptional cases. (c) Following the production of the endosteal reaction with estrogen this activity was reversed by the administration of a large dose of parathyroid extract (Bloom, McLean and Bloom, '42). (d) Results of our inadequate tests on the effect of parathyroidectomy conform best with the view that the parathyroids actively promote the resorption of endosteal bone.

SUMMARY

Formation and resorption of medullary bone were studied macroscopically in the femurs of 126 female pigeons whose age and reproductive history were accurately known. Earlier observations of others on medullary bone formation, and on a slight resorption of medullary bone during the formation of egg shells, were confirmed.

Resorption was very incomplete in femurs of eighteen birds examined at 2 to 14 days after laying. At 20 to 28 days after laying resorption was complete in only 25% of sixteen birds. The shortest interval at which complete bone resorption was observed was 22 days. Many or most pigeons of the colony studied show only partial resorption of medullary bone between successive cycles of egg-laying.

Data obtained on weight of oviducts and on diameter of follicles indicate that resorption of medullary bone occurs primarily as a result of a decrease of estrogen production by the bird. Less definite evidence suggests that parathyroid activity also may be indispensable.

Cases of hyperossification were observed in which the shaft was completely filled with hard, almost whitish bone, and the marrow spaces at the ends of the femur were partly calcified. The ability of estrogen to suppress marrow cells warrants tests of its helpful action in some of the rare and fatal blood diseases of man.

The period of hyperossification is paralleled by a period of increase of the plasma calcium. Data for changes in the plasma calcium of pigeons during the reproductive cycle are presented.

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THE OCCURRENCE OF BURST ATRETIC FOLLICLES IN BIRDS [1]

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Avian eggs which contain a large amount of yolk rupture the theca in the process of atresia. The yolk material and frequently part of the granulosa layer flow out into the stroma of the ovary, where phagocytosis occurs. This process has been described in detail (Davis, '42a) and contrasted (Davis, '42b) with the regression of the postovulatory follicle. The purpose of this note is to record the occurrence of burst atretic follicles in many avian species. The few previous records are included for completeness.

For the determination of the breeding season of birds in Brazil, the gonads of about 800 specimens were sectioned and examined. The sections were cut at 10 or 20 microns and stained in haematoxylin and eosin. I am indebted to Dr. Madureira Pará for the preparation and preliminary examination of the slides.

The ovaries were classified in five stages. All species represented in the Brazilian collection by three or more ovaries in the active or regressing phase showed the phenomenon of bursting atresia. These species, together with others previously recorded, are listed in table 1 according to families. The countries in which the specimens were collected are indicated. Previous records are noted in the third column. In all, sixty-one species, representing twenty-eight families, are named in the table.

Bursting atresia may be expected only during the period of reproductive activity. In table 2 the number of individuals containing burst atretic follicles is noted in relation to the stage of reproductive activity. These data indicate that bursting occurs most frequently in the active and regressing stages.

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[1] The work on which these observations are based was done under the auspices of the Serviço de Estudos e Pesquisas sôbre a Febre Amarela (Yellow Fever Research Service), which is maintained jointly by the Ministry of Education and Health of Brazil and the International Health Division of The Rockefeller Foundation.

TABLE 1

Avian species in which the occurrence of burst atretic follicles has been observed.

FAMILY AND SPECIES	PLACE
Tinamidae-Tinamous	
Crypturellus obsoletus	Brazil
Colymbidae-Grebes	
Poliocephalus dominicus	Brazil
Procellariidae-Fulmars	
Fulmarus glacialis[1]	Canada
Acciptridae-Hawks	
Odontriorchis palliatus	Brazil
Phasianidae-Pheasants	
Penelope obscurus	Brazil
Gallus domesticus[3]	Domestic
Laridae-Gulls	
Larus argentatus[2]	U.S.A.
Larus dominicanus	Brazil
Columbidae-Pigeons	
Columba livia[3]	Domestic
Columbigallina talpacoti	Brazil
Columbigallina minuta	Brazil
Claravis pretiosa	Brazil
Leptoptila rufarilla	Brazil
Psittacidae-Parrots	
Pyrrhura frontalis	Brazil
Pionius maximilana	Brazil
Cuculidae-Cuckoos	
Guira guira	Brazil
Crotophaga ani[2]	Cuba, Brazil
Strigidae-Owls	
Speotylo cunicularia	Brazil
Trochilidae-Hummingbirds	
Archilochus colubus[2]	U.S.A.
Trogonidae-Trogons	
Trogonurus rufus	Brazil
Bucconidae-Puffbirds	
Chilidoptera tenebrosa[2]	British Guiana
Dendrocoloptidae-Woodhewers	
Dendrocoloptes certhia[2]	British Guiana
Dendrocoloptes platyrostris	Brazil
Xyphocolaptes albicollis	Brazil
Furnariidae-Ovenbirds	
Synalaxis spixi	Brazil
Cranioleuca pallida	Brazil
Anabazenops fuscus	Brazil
Xenicopsordes amaurotis	Brazil
Sclerurus scansor	Brazil
Lochmias nematura	Brazil
Formicariidae-Ant-trushes	
Batara cinerea	Brazil
Thamnophilus caerulescens	Brazil
Grallaria varia	Brazil
Conopophagidae-Ant-pipits	
Conopophaga lineata	Brazil
Cotingidae-Cotingas	
Attila rufus	Brazil
Tyrannidae-Tyrant Flycatchers	
Muscivora tyrannus	Brazil
Empidonomus varius	Brazil
Myiodynastes solitarius	Brazil
Myiozetetes similis	Brazil
Pitangus sulphuratus	Brazil
Myiobius ridgwayi	Brazil
Oxyruncidae-Sharp bills	
Oxyruncus cristatus	Brazil
Alandidae-Larks	
Anthus lutescens	Brazil
Troglodytidae-Wrens	
Troglodytes musculus	Brazil
Turdidae-Thrushes	
Platycichla flavipes	Brazil
Coerebidae-Honey creepers	
Coerebra flaveola	Brazil
Ploceidae-Weaver-finches	
Passer domesticus	Brazil
Icteridae-Troupials	
Xanthornus decumanus	Brazil
Molothrus bonariensis[2]	Argentina, Brazil
Thraupidae-Tanagers	
Tanagra chalybea	Brazil
Thraupis ornata	Brazil
Thraupis sayaca	Brazil
Thylopsis sordida	Brazil
Schistochlamys ruficapilla	Brazil
Fringillidae-Finches	
Saltator similis	Brazil
Passerina cyanea[2]	U.S.A.
Sporophila frontalis	Brazil
Volatinia jacarina	Brazil
Junco hyemalis[3]	Canada
Zonotrichia capensis	Brazil
Haplospisa unicolor	Brazil

Species –61
Families–28

[1] Wynne-Edwards, '39.
[2] Davis, '42.
[3] Rowan, '30.

TABLE 2

The occurrence of burst atretic follicles in relation to stage of reproductive activity.

BURST FOLLICLES	NUMBER OF OVARIES EXAMINED					TOTAL
	Immature	Inactive	Progressing	Active	Regressing	
Present	0	4	11	29	28	72
Absent	57	132	46	24	23	282
Total	57	136	57	53	51	354
Per cent present	0	2.94	19.29	54.71	54.98	

SUMMARY

Burst atretic follicles were found in sixty-one avian species, representing twenty-eight families. Bursting atresia occurs most frequently in the active and regressing stages of the ovary. It is concluded that the phenomenon of bursting atresia is the usual manner of elimination of eggs which have a large quantity of yolk.

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A CYTOLOGICAL STAIN FOR THE ANTERIOR PITUITARY GLAND INVOLVING THE USE OF BASIC FUCHSIN[1]

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Several cytological stains have recently been described for the anterior pituitary gland which stain the various cell types selectively and also differentiate the mitochondria from the specific granules in both the eosinophiles and basophiles (Severinghaus, '32, and personal communication; Fain and Wolfe, '39). In these procedures Altmann's acid fuchsin, applied with heat, has been used. For the past few years we have been interested in devising a satisfactory mitochondrial technique for the anterior lobe in which the application of heat would be unnecessary. By making successive modifications of our first method mentioned above, the simple and rapid technique described below has gradually been evolved.

This technique has now been used extensively on the anterior hypophyses of rats and to a lesser extent on those of dogs. It has been found to stain the cells of the anterior lobe[2] selectively and to give a sharp differentiation between the mitochondria and the eosinophilic or basophilic granules. The procedure differs fundamentally from those mentioned above in that basic fuchsin is substituted for acid fuchsin, and it is applied without the use of heat. Good results have usually been obtained on tissue fixed either in Champy's fluid or in Regaud's fluid.

When Champy's fluid is used, the anterior lobes of mature rats are usually divided into two or three pieces and those of immature rats into two pieces. These are fixed for 24 hours, postchromated by the modified Champy-Kull method of Severinghaus ('32) and washed overnight. Much of the Champy-fixed tissue was double embedded, the procedures outlined by Severinghaus (personal communication) and by Severinghaus and Thompson ('39) being followed in detail. Good results have also been obtained by direct embedding in Tissuemat (Fisher

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[1] This study has been aided by a grant from the International Cancer Research Foundation.

[2] This procedure does not differentiate the extra cell type (Wolfe and associates, '33) in the anterior lobe of the dog which is stained selectively by erythrosin with the erythrosin, orange-G and aniline blue method of Cleveland and Wolfe, ('32).

Scientific Co.). In such instances the tissues are dehydrated during the course of 1 day and passed into cedarwood oil for clearing. They are then embedded in 60 to 62 degree Tissuemat.

When Regaud's fluid is used, the glands of mature rats are usually divided into two halves; those of immature rats may or may not be divided. They are fixed for 5 days at room temperature, the fluid being changed daily. They are then postchromated for 4 days in 3% potassium dichromate, with a change of fluid at the end of the first 48 hours. They are washed overnight, dehydrated during the course of 1 day, cleared in cedarwood oil and embedded in 60 to 62 degree Tissuemat. All sections are cut at 3 micra.

Three per cent basic fuchsin is made up by shaking 3 gm. of basic fuchsin (National Aniline Co.) vigorously in 3% aniline water. The aniline water is prepared by mixing 3 cc. of clear aniline in 100 cc. of distilled water. For either Champy or Regaud tissue, the procedures outlined below are followed.

1. Run the section down to distilled water.
2. Immerse in 3% basic fuchsin for 30 seconds to 3 minutes. Rinse in distilled water.
3. Differentiate for 10 to 30 seconds in 1% aqueous oxalic acid. Rinse in distilled water.
4. Counterstain for 1 to 15 minutes in a solution of aniline blue made up as follows:

Aniline blue	2.0	gm.
Oxalic acid	.5	gm.
Neutral formalin	2.0	cc.
Distilled water	96.0	cc.

The oxalic acid is first dissolved in the distilled water; the aniline blue is then shaken in this solution and the neutral formalin is added last.

5. Rinse, dehydrate and mount in balsam. In staining a group of sections from a single block we have found it advantageous to prepare a trial section stained for 1 minute in basic fuchsin, differentiated for 10 seconds in oxalic acid and stained for 3 minutes in aniline blue solution. The staining of subsequent sections may then be modified until the best staining time for that particular block is determined.

In sections stained by this procedure, in both the Champy and Regaud tissue, the mitochondria stain a brilliant fuchsin. The eosinophilic granules stain either a pinkish-red, orange-red or orange, and the basophilic granules moderate to deep blue. Connective tissue stains blue, and red blood cells either orange-red or red.

In the Champy tissue, the mitochondria are usually granular in nature, although short rods or filaments are sometimes seen. In the Regaud material, granular mitochondria usually predominate, although mitochondria in the form of rods and filaments are often encountered.

Certain facts about the staining of the eosinophiles are of interest. Often it was observed that the closely packed granules in many of the smaller cells show a considerable tendency to stain more intensely (more red and less orange) with basic fuchsin than do the granules in partially degranulated cells or in larger cells in which the granules are less closely arranged. It is not known whether this difference in reactivity to the stain is due merely to the fact that the granules are more closely packed in the smaller cells or whether there is some actual chemical difference in the nature of the granules which modifies their staining reaction. In either case the degree of differentiation between the eosinophilic granules and the mitochondria is more pronounced in those eosinophiles in which the granules are less closely packed, since the granules do not stain as intensely with the basic fuchsin, and hence contrast more strongly with the mitochondria which are brilliantly stained with basic fuchsin.

Although the results obtained with this staining technique have generally been good, one difficulty was met with which should be mentioned. A few blocks of tissue were encountered in which the differentiation between the eosinophilic granules and the mitochondria was either poor, or, in a very few cases, was not obtained at all. This situation was most often found in the Champy-fixed tissue. In sections from such blocks the small eosinophiles with closely packed granules usually stained intensely with basic fuchsin and the mitochondria could not be differentiated. In the eosinophiles in which the granules were less closely packed, differentiation between the granules and the mitochondria was usually present, but was often unsatisfactory. When such pieces of tissue were encountered, it was usually not possible to remedy the situation by merely varying the staining procedure. This experience has led us to the belief that the several steps in the treatment of the tissue taken prior to the actual staining have the most direct bearing on the production of successful preparations; of these steps, proper fixation is considered to be the most important.

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RENAL LYMPHATICS

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TWO TEXT FIGURES AND THREE PLATES (TEN FIGURES)

INTRODUCTION

The lymphatic system figures prominently in the theories of bacterial and malignant spread within the animal organism despite the fact that, in many organs and tissues, its anatomy and physiology are incompletely known. One of the larger gaps in this knowledge is in the field of renal lymphatics; yet it is not unusual for clinical investigators to assume the presence of lymphatics throughout the kidney and even to describe them in sections from routine autopsies (Weiss and Parker, '39). This is more surprising when one considers the markedly divergent views on many important points pertaining to the extent and distribution of the renal lymphatics. It is thus evident that a critical revaluation of the subject is desirable.

Despite general agreement in the number and course of the lymphatic vessels leaving the kidney hilus, the ultimate internal distribution has never been adequately or even convincingly described. The internal lymphatics in general accompany the blood vessels, following in succession the interlobular vessels between the medullary rays of Ferrein, the arcuate vessels along the bases of the pyramids, and the interlobular vessels in the renal columns of Bertini. Some of the cortical lymphatics communicate with the peri-renal ones. So much only is known with any degree of certainty. Lymphatics are also described by some and denied by others in the following positions: accompanying glomerular arterioles, about Bowman's capsule, in the medullary rays, in the medullary substance proper, and in the glomerulus. The precise relation of the lymphatics to the renal blood vessels has also not been completely elucidated.

Thus the question of the extent and distribution of the renal lymphatics is far from settled. Certain important difficulties, however, are inherent in any study of the terminal ramifications of the lymphatic system in a parenchymal organ. The lymphatic vessels must ordinarily be injected from within the organ outward because of the closely spaced valves located in the collecting lymphatic trunks. The widely used method of stab injections is not entirely satisfactory because, as a rule, other spaces besides the lymphatics become variably filled with the injection mass. Thus in the kidney, besides lymphatics, tissue spaces, blood capillaries, convoluted tubules, and venules may become filled to a variable and unpredictable degree, and structures so injected have been in the past mistaken for lymphatics. The criteria for a completely successful stab injection are relatively simple, but are difficult to fulfill, especially in the kidney. They comprise (1) the appearance of the injection mass in the hilar lymphatics; (2) injection of no structures other than lymphatics; (3) demonstration of anatomical continuity of all injected structures, and (4) the presence of no uninjected lymphatics in the region studied. It is rare for a preparation to satisfy even two of these criteria, so that it is necessary for the investigator to interpret somewhat ambiguous material. Because of these difficulties, we have endeavored in the present investigation not only to re-evaluate the results of previous attempts to inject the lymphatics of the kidney, but also to devise improvements in technique. In addition to methods of direct injection of the lymphatics, we have experimented with a colloidal diazo dye, trypan blue, which when injected in suitable amounts into the blood stream of a living animal passes into the lymphatics and delineates them. As applied to the kidney, this technique has yielded promising, although not entirely satisfactory, results.

MATERIALS AND METHODS

All of the present experiments were performed on living dogs, rabbits, or guinea-pigs suitably anesthetized. There was no special selection for sex, age, or weight. Most of the experiments were conducted on dogs because, in reference to size and structure, their kidneys bear a greater resemblance to the human than those of the laboratory rodents.

For stab injections, a mixture of 1 part Higgins' waterproof India ink to 2 parts of water was employed. This is believed to be as suitable as Berlin blue in water (Stahr, '00; Ssysganow, '30; Kutsuna et al., '39), and requires no elaborate preparation. The freshly filtered suspension constitutes a free-flowing mass that will penetrate the finest vessels to produce a picture suitable for microscopic study. It will not diffuse or produce vascular reactions, and is consequently suitable for in vivo as well as postmortem injections. Injections were performed with a small record syringe or with a small calibrated ink reservoir that could be raised or lowered to alter the hydrostatic pressure. A 27-gauge steel needle was found to be entirely satisfactory.

Attempts were made to inject ink under the fibrous capsule without entering the kidney tissue itself, but some penetration of the renal parenchyma usually occurred, so that these are in reality stab preparations. Other stab injections were made at various levels of the renal cortex. In most instances slabs of the tissue were fixed in 10% neutral formalin. After 2 days, the blocks were run through alcohol to benzene and finally cleared in methyl salicylate. Following preliminary examination of the pieces, promising ones were sectioned by hand, while others were run through xylol to paraffin, sectioned at 10 to 25 μ, and stained with hematoxylin and eosin. In one instance, after completing the injection with India ink, the kidneys were perfused through the aorta with Carnoy's solution for purpose of fixation.

Berlin blue in turpentine (Gerota, 1896; Bartels, '04) has been extensively used in dead material by a number of investigators in order to delineate the lymphatics in various organs (Stahr, '00; Stephanis, '02; Kumita, '09; Nicolesco, '30; Ssysganow, '30; Etcheverri, '35; Monserrat, '39). Injected in vivo it produces abnormal dilatation of the blood vessels and hence it is not nearly so satisfactory as India ink. However, it was employed in the present study in order to repeat and evaluate the work of Jasienski ('35) on the renal lymphatics.

Trypan blue was utilized for two types of preparations. In the first, a 1% solution in water or saline was injected under the fibrous capsule of the kidney to form a bleb, and after 30 to 60 minutes the dog was killed. It was hoped that the dye would be picked up by the lymphatics and form a blue protein coagulum as described by Pfuhl ('40) in the skin. These attempts, however, produced negative results. In the second procedure, a 1 to 3.5% solution in saline was injected intravenously in doses of 120 to 350 mg. per kilogram, the animal being killed 20 to 60 minutes later. This dose was not found to be lethal for dogs, guinea-pigs or rabbits, although so suggested to be by King ('38). Furthermore, the work of Wallace ('39) indicated that localized freezing with dry ice would increase vascular permeability and facilitate the transfer of dye from the blood vessels into the lymphatic system, while Lewis and Love ('26) had previously shown that quick freezing was not productive of significant necrosis or extravasation of blood cells. Consequently, in a few experiments, small areas of the kidney were treated with carbon dioxide snow or solid "dry ice." Since lymphatics are always readily demonstrable in the small intestine after suitable intravenous trypan blue injection, sections of this organ were used as controls. Slices of the kidneys and other tissues were fixed for 3 to 14 hours in "susa," then iodized, decolorized, run through alcohols and xylol, and embedded in paraffin. Sections were cut at 10 and 20 μ and studied both unstained and stained lightly with hematoxylin and eosin.

A number of other methods were considered more or less briefly and discarded as either unsuitable or too difficult. Some of these may be mentioned. Lee ('23), after completely ligating the thoracic duct in a cat, obtained a distention of the lymphatics of such magnitude as to render the valves of the hepatic lymphatics incompetent. A perfect retrograde injection of the liver with Berlin blue and water was then possible. Similar injections have been attempted by Lee as well as others for the kidney, but have never been successful, because the numerous small valves in the renal sinus (fig. 1) are apparently not rendered incompetent by the procedure. Although one thoracic duct ligation was carried out in this study in order to facilitate dissection of the hilar lymphatics, no actual retrograde injection was attempted. There are several reported injections via cannulated capsular lymphatics (Ludwig and Zawarkin, 1863; Rindowski, 1869), but the pictures so obtained have the appearance of showing either ill-defined tissue spaces or networks of blood capillaries. Several similar attempts made in the course of this investigation produced no parenchymal lymphatic injection. Various silver staining methods have been employed, but mainly for capsular lymphatics (Dogiel, 1883; Rindowski, 1869; Stahr, '00). It seems not unreasonable that careful exploitation of such methods as silver citrate perfusion (Bensley, '29) or intravenous injection of dichlorfluorescein (Bensley and Bensley, '38) might be productive of significant results. The methods, however, are difficult, and in this study the former, at least, was unsuccessful.

For completeness it should be noted that Vogel (1891) made a unique study of the renal lymphatics of a pair of kidneys showing extensive lymphatic metastases from a carcinoma of the gall bladder. It would be highly desirable to repeat his observations, but such kidneys must be excessively rare.

RESULTS AND EVALUATION OF METHODS

Successful injection of the renal lymphatics by the stab method is a matter of chance. Given a small stab and a low injection pressure, a good preparation may be obtained if the point of the needle reaches the vicinity of a large artery where the lymphatics are always abundant. Unfortunately the exact location of the arteries cannot be determined by inspection of the kidney at the time of injection. Large stabs or high pressures, on the other hand, are never satisfactory, for they produce

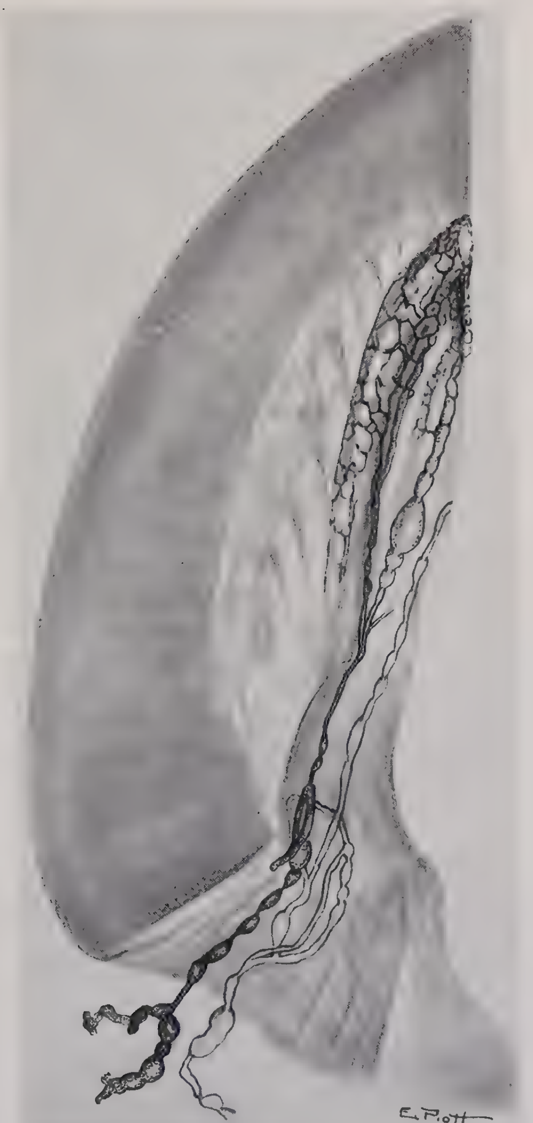

Fig. 1 Lymphatic of renal hilus. Two lymphatic trunks take origin from a lymphatic plexus about an interlobular blood vessel and pass by way of a renal column to the renal sinus. Note the numerous valves. The small lymphatics are all seen on examination of the specimen to be associated with large blood vessels. Dog kidney, stab injected with India ink in vivo, perfused with Carnoy's solution, and cleared in oil of wintergreen. Part of the pelvis and peri-pelvic fat have been dissected away. Unstained. × 6.

widespread extravasations into tissue spaces, renal tubules and blood vessels. The present series of stab injections includes the kidneys of fifteen animals, yet in only one instance was a completely satisfactory preparation obtained. Nevertheless, a portion of this particular preparation at some distance from the stab itself satisfies all four of the requirements of a successful injection as given above. Here, there is no question of the identity of the delineated structures, for they are observed to connect directly with a large, draining lymphatic trunk leaving the hilus of the kidney (fig. 1). With the exception of Etcheverri ('35), who reports three satisfactory preparations in 50 attempts, his criterion being visible injection to the regional nodes, other writers unfortunately have not presented data as to the relative incidence of success of their methods.

Of the remaining kidneys, a considerable number, when sectioned, revealed injected blood vessels which could easily be mistaken for lymphatics if the presence of a rich lymphatic plexus or network was anticipated. Moreover, many of these kidneys also showed India-ink-filled hilar lymphatics indicating that, besides blood vessels, at least some true lymphatics had been injected. The cortices of such kidneys showed areas containing characteristically irregular channels containing India ink some of which appeared to end blindly, while others seemed to enter glomeruli. Such a typical field is shown in figure 3. Similar pictures of injected channels have been regarded as lymphatics by Maximow and Bloom ('38) and others. Such an assumption seems to us to be quite unwarranted, since we have been unable to demonstrate any connections between such injected vessels and the ink-containing lymphatics at the hilus. None of the previous investigators, who have assumed that such patterns represent renal lymphatics, appears to have deemed it necessary to demonstrate rigorously the presence of connections between the channels in question and draining lymphatics. We have not been able to gain such proof from study of our own preparations, and hence we regard these questionable channels as being blood vessels. In that event, the appearance of vessels which end blindly must be attributable to occasional venules which have incompletely filled with ink, while, similarly, the threads of ink penetrating glomeruli must be interpreted as injected capillaries.

True lymphatics do accompany the major renal blood vessels as we have been able to demonstrate repeatedly. They are distinctly more numerous around the arteries than about the veins, and they follow the distribution of connective tissue septa within the kidney (figs. 4 and 5). Moreover, since the glomeruli are situated at the periphery of the renal structural units, they often abut on the connective tissue septa, and in consequence of this lymphatics may come into relatively close relationship with a certain number of them. In no case, however, has careful study of 25 μ serial sections shown these lymphatics actually entering any of the glomeruli (fig. 2). There is also repeated negative evidence to indicate that lymphatics do not occur in the medullary rays or in the medullary substance proper, since they were not found there in any of the preparations which showed rather complete filling of the perivascular lymphatic plexuses around the interlobular blood vessels. Moreover, lymphatics were never filled by a stab injection made

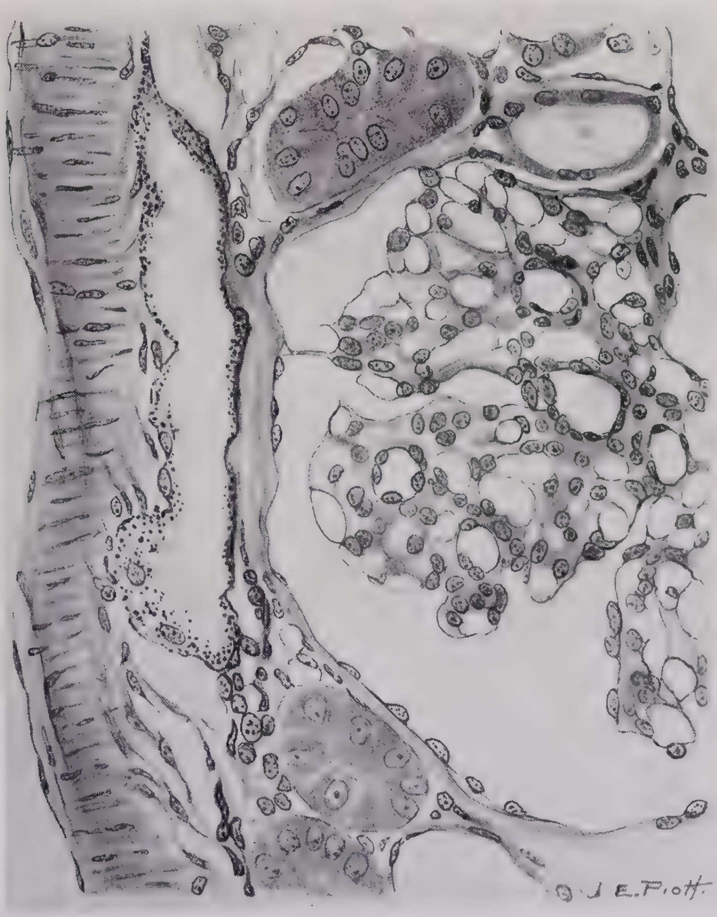

Fig. 2 Perivascular lymphatic close to a glomerulus. The lymphatic which contains granules of India ink lies between an arcuate artery and a glomerulus. Examination of serial sections shows it to be associated with the former not the latter. From the same kidney as figure 1. 25 μ. H. and E. × 540.

directly into the medullary tissue at any distance from larger blood vessels.

Trypan blue was also utilized as a means of studying the renal lymphatics. Experiments in which it was injected locally under the renal capsule were uniformly unsuccessful, but others, in which the dye was administered intravenously, did demonstrate the presence of lymphatics in the kidney, although not with the same degree of success as one can achieve in some other tissues of the body.

When large amounts of this dye in the range of 120 to 350 mg. per kilogram are injected into the circulation of a living dog, or other experimental animal, the dye quickly appears in the lymph and urine. And, within the range of this dosage and with proper timing, it is possible to demonstrate the lymphatic vessels of certain organs and tissues both grossly and microscopically. Following this procedure, the abdominal lymphatics, including those of the intestine, liver and kidney, become well outlined by virtue of the trypan blue within them. The lymphatics can be perceived grossly as blue channels, while in histological sections they can be recognized by the presence within them of blue coagulum or precipitate (figs. 6, 8 and 10). A perfect preparation of this kind should give a maximal color density in the lymphatics and a minimal coloration of the blood. The principal variables consist in the amount of dye administered and the interval between its injection and the time at which the animal is killed.

The diffusion of the dye from blood vessels may be hastened and its passage into the lymphatics increased locally by application of solid carbon dioxide to the surface of certain organs. In the intestine this procedure definitely augments the amount of trypan blue in the intrinsic as well as extrinsic lymphatics of this organ and makes them more readily visible. This procedure induces definite hyperemia and some edema in the wall of the intestine, but no apparent necrosis or extravasations of blood cells from the circulation during the period of less than an hour during which the experiment lasts.

Unfortunately these methods yielded less satisfactory results in the case of the kidney than in the intestine. Following massive intravascular injections of trypan blue the renal lymphatics did concentrate the dye, although not to the same degree or with the same intensity as in the control tissues. The relative intensity of staining of the lymphatics in several organs, including the kidney, is illustrated in figures 6, 8, 9 and 10. The lymphatic channel shown in figure 10 is from the hilus of a mesenteric lymph gland and shows the greatest concentration of trypan blue. The large lymphatic trunk from the serosal coat of the intestine, in figure 6, displays an intermediate coloration, whereas the renal lymphatics visible in figures 8 and 9 contain considerable less trypan blue as indicated by the fact that they are paler.

In spite of the disappointingly low intensity of the dye in the renal lymphatics, the lymphatic channels there could be followed to a considerable extent in 20 μ serial sections. As by the India ink injection method, perivascular plexuses draining into lymphatics at the hilus could be easily identified and followed (figs. 8 and 9). Lymphatics surrounding the blood vessels in the cortical interlobular septa could be seen and these sometimes occurred close to glomeruli (fig. 9), but none could be traced into the glomeruli or the renal lobules. It must be confessed, however, that the trypan blue in the smallest lymphatics was relatively pale, and we cannot claim categorically that we were able to identify or account for all of the smallest possible lymphatic radicals. Yet, as was the case after injection with India ink, with this method also no lymphatics were found entering the medullary tissue.

In the case of the kidney we tried also to increase the concentration of trypan blue in the lymphatics by freezing portions of the renal cortex with dry carbon dioxide. The results in the kidney differed from those obtained in the intestine, which was used as a control, and were unsatisfactory as far as delineation of lymphatics was concerned. The freezing did not noticeably augment the passage of dye from the renal blood vessels into the lymphatics. Moreover, within the span of an hour's time, the epithelium of the renal tubules began to show visible necrosis accompanied by diffusion of trypan blue into the necrotic cells with resultant staining of their nuclei (fig. 7). As in the case of the intestine, hyperemia was induced, but contrariwise, there was little or no evidence of edema, a circumstance which we attribute to the relative paucity of connective tissue in the kidney and to relative absence of capsular distensibility.

Finally, we essayed to demonstrate the renal lymphatics by immediate postmortem introduction of Gerota's mass by stab injections into the kidney. Under these conditions the mass has a tendency to fill vessels unevenly and to produce a distorted picture. Instead of forming a film evenly coating the interior of the injected vessels and tubules, the mass tends to form threads and droplets which frequently give the appearance of fine anastomosing patterns identical with the excessively fine and rich renal lymphatics described by Jasienski ('35). A typical field of such a preparation is shown in figure 11. From study of our material prepared by this method, it appears evident that the structures accepted as lymphatic networks by Jasienski are in reality artifacts.

DISCUSSION

Since disagreement as to the distribution of lymphatics in the kidney parenchyma centers about the finer vessels, it seems desirable to present a brief summary of the literature.

1. Rindowski (1869) and Kumita ('09) maintain that the afferent and efferent arterioles of the glomeruli are accompanied by lymphatics, but Vogel (1891) insists that no such lymphatic channels are demonstrable.

2. Rindowski, Vogel, Kumita, and Ssysganow ('30) describe an extensive network of lymphatics about Bowman's capsule. Stahr ('00) denies the presence of lymphatics there, while Kutsuna et al. ('39) state merely that lymphatics come close to the capsule without forming a network.

3. Rindowski, Vogel, Kumita, and Ssysganow describe regular lymphatic capillary plexuses in the medullary rays of Ferrein, but their presence in that location is considered doubtful by Stahr and Kutsuna et al.

4. Rindowski describes single medullary vessels dividing dichotomously. Kumita maintains that there is an extensive lymphatic meshwork similar in extent and distribution to the capillary bed, while Ssysganow describes two such medullary lymphatic plexuses. In distinct opposition to these writers are again Stahr and Kutsuna et al. who find the presence of medullary lymphatics infrequent and questionable.

5. The literature is sharply divided on the subject of lymphatic entry into the glomerulus. Rindowski, Kumita, Maximow and Bloom ('38), and Ssysganow describe such penetration, while Stahr, Kutsuna et al. and Drinker and Yoffey ('41) are opposed to the idea. None of the descriptions of glomerular lymphatics is adequate, and consequently their existence is definitely unproven.

In summary of our own experience it may be stated that the only renal lymphatics which we have been able to demonstrate to our own satisfaction are those which surround and accompany the interlobular, arcuate and interlobar vessels. These plexuses connect mainly with draining lymphatics in the hilus of the kidney, but they possess connections as well with occasional efferent capsular lymphatics. We have not been able to convince ourselves of the presence of lymphatics entering the glomeruli, forming a network about Bowman's capsule, or lying in the medullary rays as others have claimed. We regard the structures interpreted as lymphatics in these localities as being artifacts produced by partial injections of capillaries, venules, tissue spaces or even in some instances renal tubules. Certain of the patterns so produced have a similarity to lymphatics, but we have not been able from carefully following some of these in serial sections to demonstrate that they connect directly with the true lymphatics which occur in the interlobular spaces and hilus of the kidney. In the absence of such proof, as well as for the reasons presented in the text, we regard lymphatics as non-existent in the localities cited.

Our conclusions are compatible with certain ideas pertaining to kidney function and structure. The lymphatic vessels in parenchymatous organs are in general related to structural units. As Lee has shown for the liver ('23) the lymphatics of organs which are built on a unit principle do not enter the substance of the lobules but remain confined to the perilobular connective tissue. This has been our experience, too, in the kidney. Traut ('23), following Peter ('09), found that maceration caused a natural cleavage of the kidney substance into radiating fasciculi (not pyramids) having firm attachments only at their apices. These Traut defined as the structural units or lobules. They are separated by blood vessels, connective tissue, and the known lymphatics. As in the liver, in the kidney, too, no lymphatics apparently actually enter these lobules. On coronal section, the renal cortex presents a series of lobules each composed of a peripheral circle of glomeruli which surrounds a central bundle of straight tubules and collecting ducts (fig. 12). In the connective tissue surrounding and uniting these lobules the major blood vessels and the only lymphatics which we have been able to demonstrate are lodged.

Another probable determinant of lymphatic distribution in the kidney relates to the fact that lymphatics in general are found only in regions relatively rich in connective tissue. According to Pullinger and Florey ('35), collagenous and reticular fibers are inserted directly into the walls of lymphatic capillaries thus providing for dilatation rather than compression of these structures in edema. Such a relationship, if generally true, would imply the presence of sufficient connective tissue around the lymphatic radicles to provide such support. At any rate, in the various organs where their presence is known, lymphatics appear to avoid regions where connective tissue is sparse, and for this reason one should probably not anticipate finding them within the renal structural units and more especially within the glomeruli.

Connective tissue is known to be present in the glomerulus (von Möllendorff, '28; Bensley and Bensley, '30; Zimmermann, '33; and Bargmann, '38), but it is exceedingly sparse except in the immediate vicinity of the vascular pole where it may occasionally be seen without special methods. Moreover, lymph capillaries are known to be larger than blood capillaries so that a not inconsiderable space should be necessary for them if any were present in the glomerulus. Obviously, it would be only in the connective tissue spaces between the blood capillaries of the glomerulus that lymphatic capillaries could exist, but, since the connective tissue is so extremely sparse, these tissue spaces are exceedingly small. Hence, the probability of lymphatics existing there seems very remote.

Concerning the question of glomerular lymphatics, Drinker and Yoffey ('41) conclude that there is no reliable evidence that any of the kidney lymph comes from the glomerular capillaries and that it would seem more probable that lymph capillaries are not found in the glomeruli and that all the renal lymph comes from extraglomerular regions. Our own observations and reasoning support their conclusion.

The relationship between renal blood vessels and lymphatics seems unusual and has not been commented on previously. The present study shows that lymphatics accompany the arteries rather than the veins. Since this is the reverse of what has been described in other regions of the body, it suggests a functional relationship, the simplest and most direct possibility being that the lymphatics collect and serve to drain fluid from the adventitia of the arteries. This idea is given some support by a number of considerations. Arteries are certainly not impermeable to fluid, solutes, and protein from the blood. Their intima and media contain no important blood capillaries, and the position in which well-known degenerative arteriosclerotic changes begin suggests that a considerable portion of the nourishment of the arterial wall is derived by diffusion from the interior of the vessel itself. Moreover, experiments described by Benninghoff ('30) show that the arterial wall can be adequately nourished even though the adventitia is completely stripped and the media coated with lanolin and wax. He showed also that in similar situations, if a dye is injected into the blood stream, it soon penetrates to the periphery of the isolated segment of the vessel wall. This is quite reasonable for arterial filtration pressures are high.

The tremendous amount of blood going through the kidneys, which according to Smith ('39) is one-third the resting cardiac output, might presumably give rise to a certain amount of peri-arterial tissue fluid. Moreover, since the arteries are the only renal structures not in close contact with the rich capillary beds of the tubules (fig. 5), it would seem reasonable to expect that the lymphatics might serve to drain this otherwise relatively isolated tissue fluid. Since kidney lymph is not large in volume (Schmidt and Hayman, '29), this might possibly represent the sole renal lymphatic function. Lee ('22), similarly, described rich lymphatic capillary plexuses situated in the adventitial coat of the aorta, which are quite separate from the larger lymph trunks traversing the region and which probably account for local drainage of tissue fluid from the arterial wall.

The kidney is one of the most stable of organs as far as its blood supply is concerned. Normally about 99% of the glomerular filtrate is believed to be reabsorbed into the tubular capillaries. In cases of trauma, or inflammation when tissue fluid is increased beyond the point at which it can be reabsorbed by the blood capillaries, there is cessation of renal function followed by cellular degeneration and necrosis. Hence, edema, even though it be slight, is apparently disastrous to the kidney, and the failure of this organ to meet such an abnormal and infrequent situation might well be attributable to the fact that there are very few lymphatics present to take over as emergency units. This is entirely different from the skin, intestine, or uterus (Wislocki and Dempsey, '39) where the lymphatics are abundant and capable of exercising an important emergency function.

SUMMARY

1. Successful lymphatic injections by the stab method are rare, purely a matter of chance, and seldom give a clear picture. The use of an intravenous dye is described as a potentially suitable method for the demonstration of parenchymal lymphatics.

2. Lymphatic distribution in the kidney is related to the structural unit which itself is determined by the distribution of the larger blood vessels and of connective tissue.

3. The lymphatics accompany the interlobular, arcuate, and interlobar vessels, surrounding them in an irregular anastomotic net. The peri-arterial network is much richer than the perivenous one. The importance of this relation for renal physiology and pathology is discussed.

4. The cortical and perirenal lymphatics connect.

5. Since the glomeruli abut directly on the connective tissue septa about the major vessels, lymphatic capillary networks are seen in the immediate vicinity of the Bowman's capsules of some glomeruli, but cannot be said to have more than a casual relationship to these structures.

6. There are probably no lymphatics in the medullary rays or in the medullary substance proper.

7. Lymphatic entry into the glomerulus would be surprising from both the structural and functional standpoints. No such entry could be demonstrated in the present study.

8. Valves are lacking in the lymphatics of the renal parenchyma, but are present in the large trunks of the renal sinus. Retrograde injection is not feasible because of these valves.

To Dr. Allan L. Grafflin who inspired this work and was helpfully critical of its progress the author wishes to express his gratitude.

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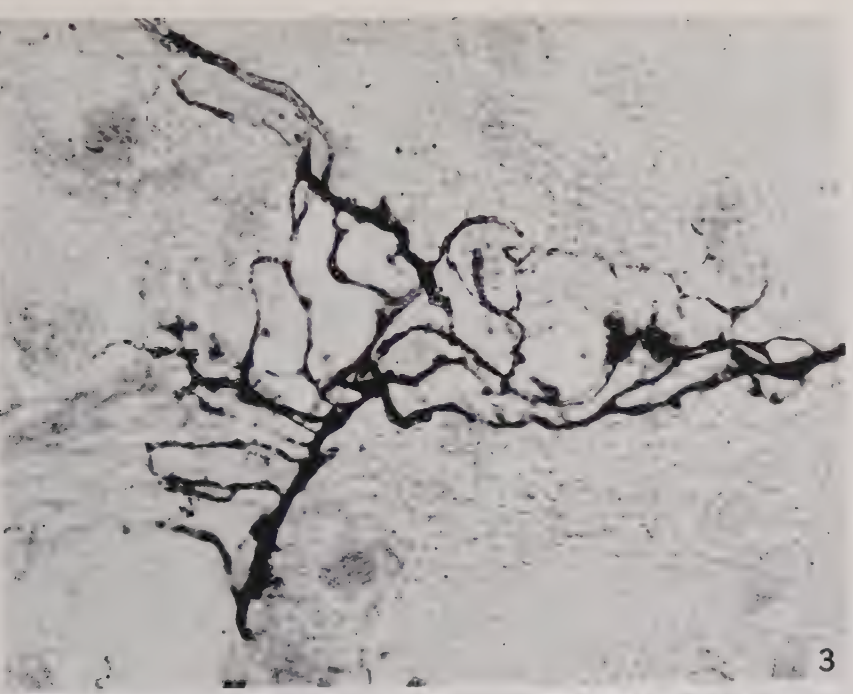
PLATE 1

EXPLANATION OF FIGURES

3 Lymphatic like pattern in kidney cortex. Note what appear to be blind ending vessels and vessels entering glomeruli. Dog kidney. India ink stab injection. Freehand section from oil of wintergreen. Unstained. × 60.

4 Lymphatic vessels around interlobular blood vessels. The lymphatics are much more abundant about the artery than about the vein. From the same kidney as figure 1. Freehand section; oil of wintergreen. Unstained. × 50.

5 Lymphatics surrounding arcuate blood vessels. Note, as in figure 4, that peri-arterial lymphatics predominate. From the same kidney as figure 1. 25 μ. H. and E. × 65.



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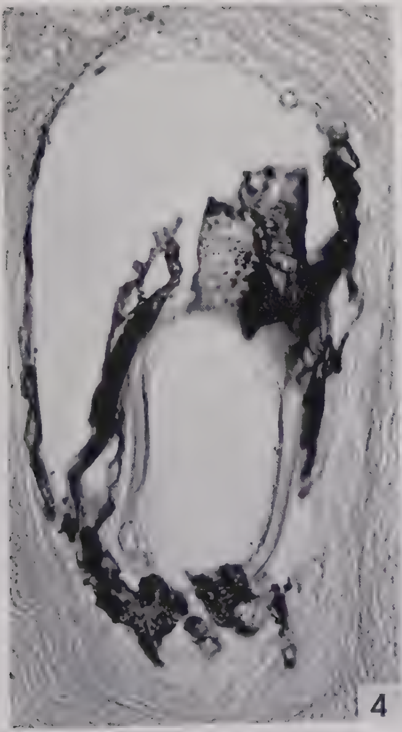

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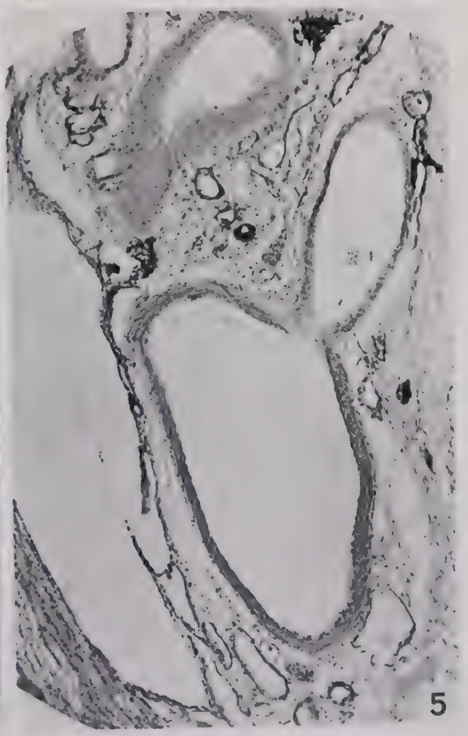

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PLATE 2

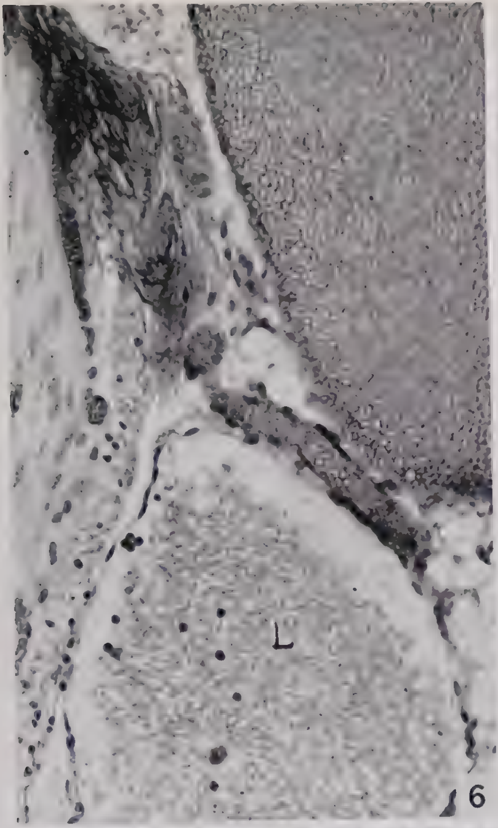
EXPLANATION OF FIGURES

6 Lymphatic vessel in subserous layer of small intestine containing a moderately dense trypan blue coagulum. (L) The connective tissue is stained darkly. Guinea-pig injected intravenously with trypan blue. 20μ. H. and E. × 220.

10 Lymphatics in a mesenteric lymph node containing a dense trypan blue coagulum in one and lighter trypan blue coagulum in several others. Rabbit injected intravenously with trypan blue. 20 μ. Unstained. × 65.

7 Changes produced in kidney cortex by rapid freezing (see text). Necrosis of tubular epithelium with nuclear staining, moderate hyperemia, and edema limited to the connective tissue capsule are well shown. Dog injected intravenously with trypan blue; kidney treated locally with solid carbon dioxide. 20 μ. Unstained. × 145.

12 Normal kidney architecture shown in coronal section. The lobular markings are accentuated by virtue of trypan blue concentrated in the proximal convoluted tubules. These and the glomeruli are seen at the periphery of the lobules, while the collecting ducts are centrally located. Rabbit killed 48 hours after intravenous injection of trypan blue (230 mg. per kg.). 20 μ. H. and E. × 65.



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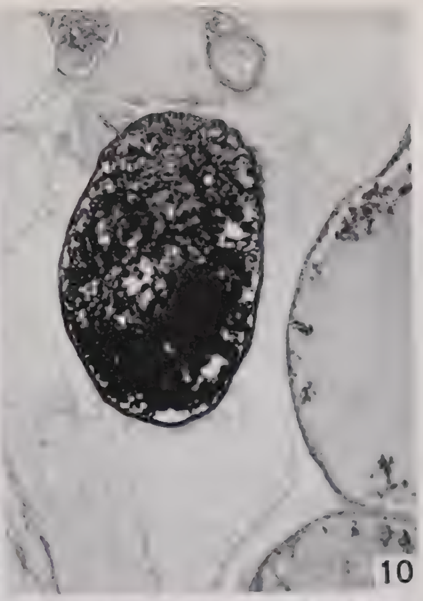

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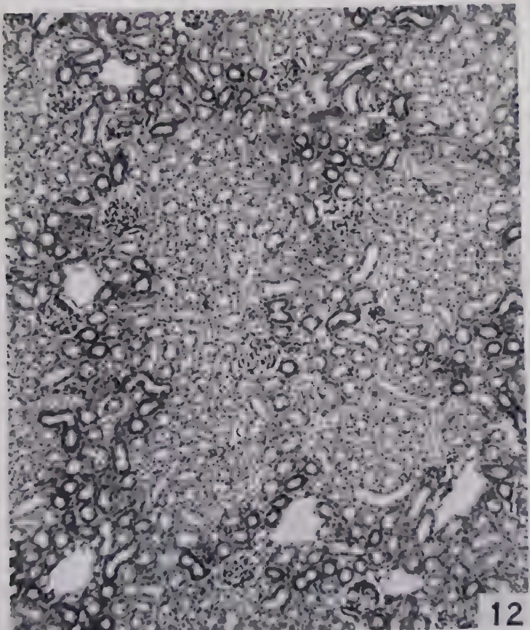

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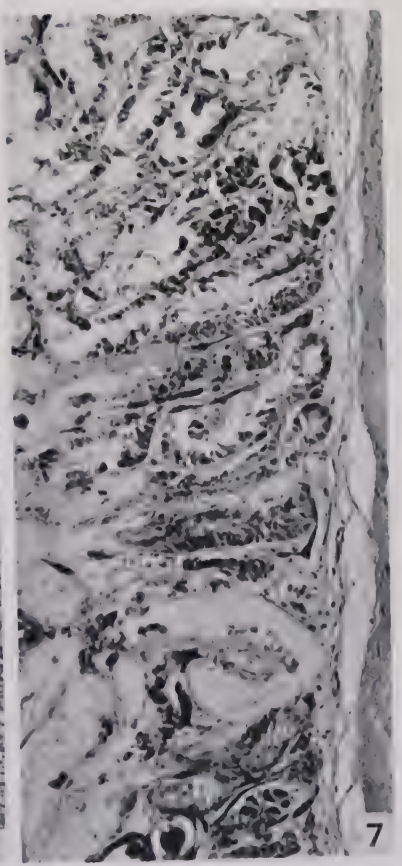

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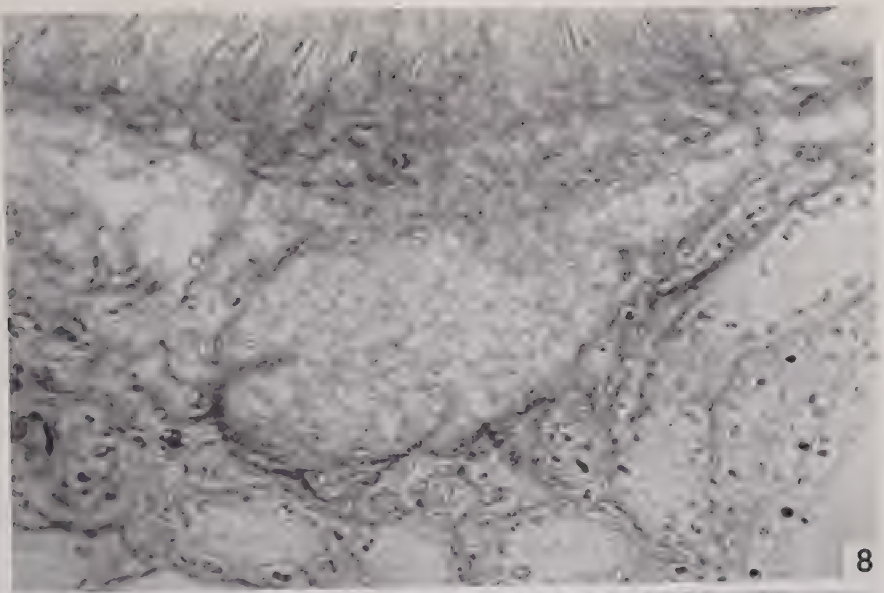
PLATE 3

EXPLANATION OF FIGURES

8 Cortical renal lymphatic in adventitia of arcuate artery containing trypan blue coagulum. Compare with figures 6 and 10 and note the connective tissue staining. Dog injected intravenously with trypan blue. 20 μ. Unstained. × 350.

9 Section of kidney showing five peri-arterial lymphatics containing trypan blue coagulum. (L) Note proximity to tubules and to a glomerulus. Guinea-pig injected intravenously with trypan blue. 20 μ. H. and E. × 520.

11 Kidney showing patterning which results when Gerota's mass is employed in vitro. Note the appearance of fine anastomosing vessels so tiny that five or six can lie between two adjacent tubules. Rabbit stab injected with Gerota's mass approximately 6 minutes after death. 25μ. Unstained. × 435.



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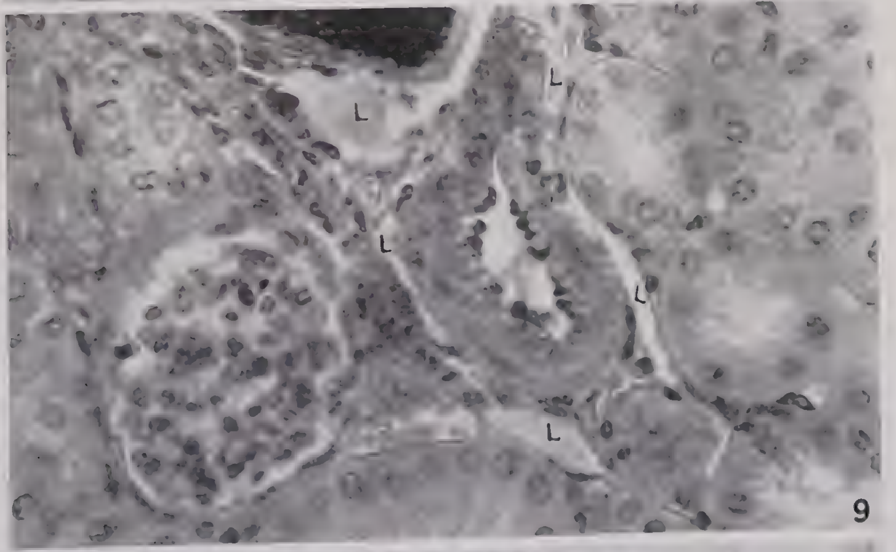

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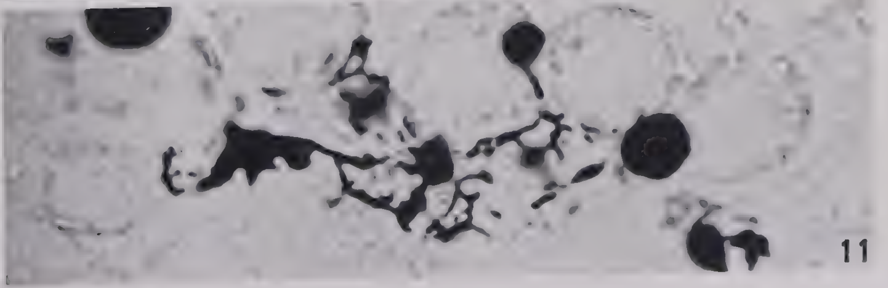

11

THE EXTRUSIVE GROWTH AND ATTRITION OF THE INCISOR TEETH OF ERETHIZON DORSATUM

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This study of the extrusive growth and attrition of the incisors of the North American Porcupine Erethizon dorsatum is one of a series of similar studies on various Rodents and Lagomorphs which were begun by the senior author. The first paper on the domestic rabbits (Shadle, '36) was followed by one on Rattus norvegicus (Shadle, Wagner and Jacobs, '36) and by one on Cavia cobaya (Shadle, Valvo and Eckhert, '38). More recently, Huruhata ('40), using a similar procedure and apparatus, has studied the Formosan wild rat, Rattus losea (Swinhoe).

When a sufficiently representative series of studies has been completed, a careful study of the results should shed considerable light on the phenomenal growth and attrition of the incisors in Rodentia and Lagomorpha.

MATERIAL

The animals used in this study were Erethizon dorsatum dorsatum and Erethizon d. epixanthum (Anderson and Rand, '43). Several of the specimens of E.d. dorsatum were taken in Allegany State Park, N. Y., some were collected in New England and others were obtained in the western Great Lakes region. The two female specimens of E.d. epixanthum were from Utah.

Of the eighteen animals used in this study, ten were males and eight females. When subjected to the experiment, their ages varied greatly; thirteen of them were at least 1 year old and several were 2 to 4 years old; four others were only a month old when the data were started and one was born and bred in the laboratory (Shadle and Ploss, '43).

ACKNOWLEDGMENTS

The authors are greatly indebted to Mr. Donald Spencer of the Fish and Wildlife Service and to Mrs. Spencer for specimens and also for valuable suggestions and observations on the porcupines. Of the animals which they raised by hand, three were 1 year of age and three

others were about 4 weeks old when they were sent to join the porcupine colony at the University of Buffalo.

For the specimens of yellow-haired porcupines, we are much indebted to the U. S. Fish and Wildlife Service and particularly to R. Scott Zimmerman at Denver, Colorado.

The Allegany State Park Commission of Red House, New York, has been very generous in giving permission for the taking of specimens in the park area, and has also been very helpful in other ways in furthering research in the area.

APPARATUS AND METHODS

The apparatus and methods of marking the incisor teeth have already been fully described (Shadle, '36, p. 16). Measurements of the marked incisors were made each week to determine the amount of attrition from each tooth during that time. New transverse marks across the labial surface of each tooth were made when necessary, which amounted to once in every 2 to 4 weeks. The weekly attrition measurements were made from this transverse line to the labial cutting edge of the tooth. In the data which follow, figures for the individual incisors are written as if one were looking directly at the teeth from a frontal view; depending upon the arrangement and position of the comparative figures, i.e. $\frac{1.40 \mid 1.41}{1.85 \mid 1.82}$ mm. means

$$\frac{\text{Upper right incisor 1.40 mm.} \mid \text{Upper left incisor 1.41 mm.}}{\text{Lower right incisor 1.85 mm.} \mid \text{Lower left incisor 1.82 mm.}}$$

The horizon line always indicates the occlusal plane, and the perpendicular line indicates the plane of division between the right and left halves of the dentition. The figures always indicate millimeters unless otherwise stated.

The actual number of days during which attrition measurements were made on each porcupine represents the individual measure of "time" for each animal, or the "Porcupine days" of "extrusive growth and attrition" (Shadle, 36, op. cit.) involved. The total number of "porcupine days" for all the animals amounts to 6258 days of data taken, or 17 years, 1 month and 23 days. Data during such a period of time, from a representative group of young, middle aged and some old animals, should give a fair indication of the extrusive growth and attrition rates of the species, under the existing conditions.

The data time of the five young animals less than a year old, totaled 1013 days, i.e. 2 years, 9 months and 13 days, or 16.2% of the total time.

RESULTS OBTAINED

First the data for each individual were computed, then the data were assembled according to (a) sex, (b) age and finally (c) a composite total figure of the group was derived.

The data for the ten males showed that during 481.4 weeks (3370 days, or 9 years, 2 months, 25 days) there was a total extrusive growth and attrition of $\frac{649.2 \mid 671.9}{836.0 \mid 884.3}$ mm., which is equivalent to an average weekly rate of $\frac{1.35 \mid 1.39}{1.73 \mid 1.83}$ mm., i.e., an average weekly amount of 6.30 mm. This amounts to a computed annual extrusive growth and attrition of $\frac{70.20 \mid 72.28}{89.96 \mid 95.16}$ mm., i.e., 327.60 mm. of incisor teeth per year for males.

The eight females during 411.9 weeks (2883 days, or 7 years, 10 months, 28 days) wore away a total of $\frac{490.7 \mid 512.2}{685.4 \mid 692.1}$ mm. This is an average weekly rate of $\frac{1.19 \mid 1.24}{1.66 \mid 1.68}$ mm., i.e., an average weekly amount of 5.77 mm. or a computed annual extrusive growth and attrition of $\frac{61.88 \mid 64.48}{86.32 \mid 87.36}$ mm., i.e., 300.04 mm. of incisor teeth per year for females. Thus the male shows 27.56 mm. greater average annual extrusive growth and attrition than the female.

The entire group of eighteen porcupines, during the 893.2 weeks (6253 days, or 17 years, 1 month, 18 days) produced and wore away the incisors as follows: $\frac{1139.9 \mid 1184.1}{1521.4 \mid 1576.4}$ mm., a weekly average of $\frac{1.28 \mid 1.32}{1.69 \mid 1.75}$ mm., i.e., 6.04 mm. of average weekly extrusive growth and attrition. This is a computed average annual amount of $\frac{66.04 \mid 68.64}{87.88 \mid 91.52}$ mm., i.e., a total of 314.08 mm., per individual, for this group.

The lower incisors are worn away faster and in considerably greater amounts than the upper incisors. The attrition index, i.e., growth and attrition expressed in the percent by which the lower incisors exceed that of the upper incisors, is shown in table 1.

TABLE 1

Summary of extrusive growth and attrition.

GROUP	TOTAL ATTRITION OF BOTH UPPER INCISORS AND BOTH LOWER INCISORS		EXTRUSIVE GROWTH AND ATTRITION INDEX, I.E., PERCENTAGE GREATER ATTRITION OF LOWER INCISORS OVER UPPER INCISORS	TIME				
	mm.	*inches*	*per cent*	*wks.*	*days*	*yrs.*	*mos.*	*days*
Males	$\frac{1321.1}{1720.3}$	$\frac{52.0}{67.8}$	23.3	481.4	3370	9	2	25
Females	$\frac{1002.9}{1377.5}$	$\frac{39.5}{54.2}$	37.3	411.9	2883	7	10	28
Total Group	$\frac{2324.0}{3097.8}$	$\frac{91.5}{122.0}$	33.3	893.2	6253	17	1	18

DISCUSSION

The incisors of the porcupine are markedly longer and larger in diameter than the incisors of the rabbits, the rats, or the guinea pigs which have been previously studied. A comparison of the extrusive growth and attrition of the porcupine with the above species shows, that the larger incisors of the porcupine, grow less rapidly, and wear away less rapidly than any one of the other three forms.

The male porcupines produce and wear away a greater amount of incisor tooth than the females do, a condition which was also found in the male guinea pigs. In both species, the males, when excited sexually, and during anger or other disturbances, have a greater tendency to chatter the teeth. Although both sexes, and all ages of both porcupines, and guinea pigs have the habit of chattering the teeth, the reaction is much more marked in the mature males of both species, and it is believed to be an important factor in the greater attrition of the incisors of the males of these species. Once the female porcupines have become accustomed to cage life, they tend to be more quiet and friendly than most of the males. The females of both albino rats, and of the domestic rabbits, had a higher attrition rate than the males. Huruhata, however, found no appreciable difference in rates of extrusive growth and attrition of the males and females of the Formosan wild rat.

In the mature porcupines, the lower incisors produced and wore away only 33.3% greater length of tooth than the upper incisors. This same comparison among the mature animals of other species was: albino rat 40%, Formosan wild rat 40%, guinea pig 25%, and the domestic rabbit 25%. From the above figures, comparing the rate of extrusive growth and attrition of small species having small incisors with larger species having larger incisors, it is evident that the incisors with the smaller diameters wear away more rapidly than the incisors with the larger diameters, in spite of the differences in size of the animals compared.

During the period of observation, one female (Maude) bred and gave birth to one young (Shadle and Ploss, '43). Comparisons of the weekly rates of the extrusive growth and attrition of the individual incisors of this female, show a marked reduction of these rates during pregnancy. This reduction was to some extent evident also during lactation.

SUMMARY

For this group of laboratory porcupines, the following points seem warranted:

1. During the accumulated total of 893.2 weeks (6253 days, or 17 years, 1 month and 18 days) of observation and measurement, sixteen

Erethizon d. dorsatum and two E. d. epixanthum studied, show average weekly extrusive growth and attrition rates of the individual incisors of $\frac{1.28 \mid 1.32}{1.69 \mid 1.75}$ mm.

2. During the above total time, the eighteen porcupines produced and wore away a total of $\frac{1139.9 \mid 1184.1}{1521.4 \mid 1576.4}$ mm., or 5.4218 meters of incisor tooth structure.

3. The computed average annual amounts per porcupine are $\frac{66.04 \mid 68.64}{87.88 \mid 91.52}$ mm. which is equivalent to a total annual production of incisors of 314.08 mm. that is, $\frac{2.6 \mid 2.7}{3.5 \mid 3.6}$ inches, or 12.4 inches of incisor teeth extruded and worn away per year.

4. Male porcupines have 27.56 mm. higher computed annual extrusive growth and attrition in the incisors than the females have. The more marked habit of the male porcupines of chattering the teeth when disturbed or sexually excited, is believed to be an important factor in the greater attrition of the incisors of the males. In all the porcupines the right incisor usually exceeded the extrusive growth and attrition of the left incisor.

5. The lower incisors of adult porcupines exceed the extrusive growth and attrition of the upper incisors by 33.3%.

6. The weekly extrusive growth and attrition rates were reduced during the single period of pregnancy and lactation observed.

7. The rate of extrusive growth and attrition of the incisors varies inversely as the diameter of the cross section of the tooth; i.e., the smaller incisors of small species of Rodentia and Lagomorpha usually show a more rapid extrusive growth and attrition than the larger incisors of larger species of Rodentia and Lagomorpha.

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